VOLUME 3 AUGUST 1957 NUMBER 5

Canadian Journal of Microbiology

Published by THE NATIONAL RESEARCH COUNCIL
OTTAWA CANADA

CANADIAN JOURNAL OF MICROBIOLOGY

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Canadian
Journal of Microbiology

Issued by THE NATIONAL RESEARCH COUNCIL OF CANADA

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MANOMETRIC STUDIES WITH RHIZOSPHERE AND NON-RHIZOSPHERE SOIL[1]

H. KATZNELSON AND J. W. ROUATT

Abstract

Manometric studies were carried out on the metabolic activity of different rhizosphere and non-rhizosphere soils from the greenhouse and the field. Oxygen consumption was distinctly greater with the rhizosphere soils. On the addition of substrates such as casamino acids or a mixture of carbohydrates and organic acids, greater oxygen uptake again occurred with the rhizosphere soils and was particularly striking with the amino acids. Chromatographic studies of extracts of rhizosphere and non-rhizosphere soil incubated with casamino acids suggested that certain amino acids such as arginine, proline, and tyrosine may be preferentially utilized in the rhizosphere.

Introduction

It has been amply demonstrated that the root zone of plants (the rhizosphere) harbors a microflora which is physiologically more active than that of soil apart from the root (6, 8, 10, 12, 14). This was done in most instances by isolating and testing large numbers of soil bacteria or by ultimate dilution procedures in differential media. These methods are time-consuming and cumbersome and frequent efforts have been made to circumvent them. During recent years attention has turned to the application of manometric methods to soil microbiology as a convenient and comparatively rapid means of determining the over-all activity of the soil under optimum conditions of moisture and temperature (1, 2, 7, 11, 13). It was considered desirable therefore to apply this technique to an investigation of soil obtained from the rhizosphere of different plants and soil a short distance from the roots, using oxygen consumption as a measure of activity. It has been suggested by Chase and Gray (2) that oxygen uptake is a better criterion of the metabolic potential of a soil than CO_2 evolution. The influence of soil treatment on the microbial activity in both rhizosphere and non-rhizosphere soil also may be evaluated readily by this method. Since it has been shown (9) that amino-acid-requiring bacteria are favored in the rhizosphere and that ammonifying bacteria are also selectively stimulated (4, 6), casamino acids were used as

[1]Manuscript received March 13, 1957.
Contribution No. 437 from the Bacteriology Division, Science Service, Canada Department of Agriculture, Ottawa.

[The previous number of Can. J. Microbiol. (3, 533-671) was issued June 25, 1957.]

one substrate. For comparison, a mixture of non-nitrogenous substances including mono- and di-saccharides, pentoses, and organic acids was also used. The ability of different soils to metabolize these substances as well as amino compounds has been reported previously (7).

Methods

Soil and root samples were obtained from greenhouse plots and from the field, the soil samples being taken at a distance of 8 – 10 inches from the plant roots. The plants themselves were dug up carefully and loosely adhering soil gently shaken off and discarded. The soil remaining on the roots was then dislodged by more vigorous shaking and collected. This constituted the rhizosphere sample. The soils were spread out in a thin layer on paper in the laboratory and dried quickly for 30 – 45 minutes with the aid of a fan. They were then passed through sieves and the 1 mm. fraction used. The moisture-holding capacity was determined and 4 g. samples weighed into Warburg flasks. Sufficient water or solution of substrate was added to bring the soil to 70% of its moisture-holding capacity; alkali (0.2 ml. 20% KOH) was added to the center wells and the vessels attached to the manometers and placed in a water bath maintained at 30° C. After a 30 minute equilibration period, the manometers were closed and readings begun; the flasks were not shaken. Two substrates were compared: casamino acids at 20 mg. per vessel and a non-nitrogenous mixture composed of 0.1 ml. (10 μM.) each of $M/10$ solutions of glucose, fructose, sucrose, maltose, xylose, arabinose, succinate, malate, acetate, and pyruvate. All experiments were carried out in duplicate.

Results

In a preliminary trial to determine the degree of variation between samples, three composite samples were taken from barley and rye plants grown in adjacent plots in the field and from control soil between the plots. The results shown in Table I indicate reasonably good agreement among the replicates.

TABLE I

OXYGEN UPTAKE, μL. BY THREE COMPOSITE SAMPLES OF RHIZOSPHERE AND NON-RHIZOSPHERE SOILS*

Composite sample	Rhizosphere soil		Control soil
	Barley	Rye	
1	359	295	101
2	363	327	101
3	392	294	96
Average	371	305	99

*After 9 hours at 30° C.; each figure is an average of duplicate determinations.

The results of experiments with a variety of crops grown in the greenhouse and in the field are illustrated in Figs. 1–3. Rhizosphere soil was distinctly more active than non-rhizosphere (check) soil (Fig. 1) except in the case of oats grown in the greenhouse; this anomalous behavior of oats has been observed previously and warrants further investigation (6, 10).

Greater oxygen consumption occurred on the addition of substrates as may be seen in Figs. 2 and 3, which represent data after subtraction of oxygen uptake by corresponding unamended soil. By the end of the experimental period, check soil treated with casamino acids was found in all cases to be more active than soil treated with the non-nitrogenous mixture. The addition of substrate to rhizosphere soil again stimulated activity especially when the amino acids were used. Certain differences in the behavior of greenhouse and field soils also may be observed. The response to non-nitrogenous materials is greater in the greenhouse soil and the over-all attack on the substrates in general appears to be more rapid, as a lag of three or more hours is noticeable with the field soils. Such differences between soils have also been observed previously (7) and require further elucidation.

The greater activity of the soils supplemented with casamino acids prompted studies to determine if certain amino acids were preferentially utilized. Casamino acids were added to both rhizosphere and non-rhizosphere soil in Erlenmeyer flasks and maintained for 10 hours in a water bath at 30° C. Amino acids were extracted twice with 70% ethanol and suitable aliquots spotted on Whatman No. 1 filter paper for descending chromatography with *n*-butanol – acetic acid – water as solvent. After 20 hours the paper was

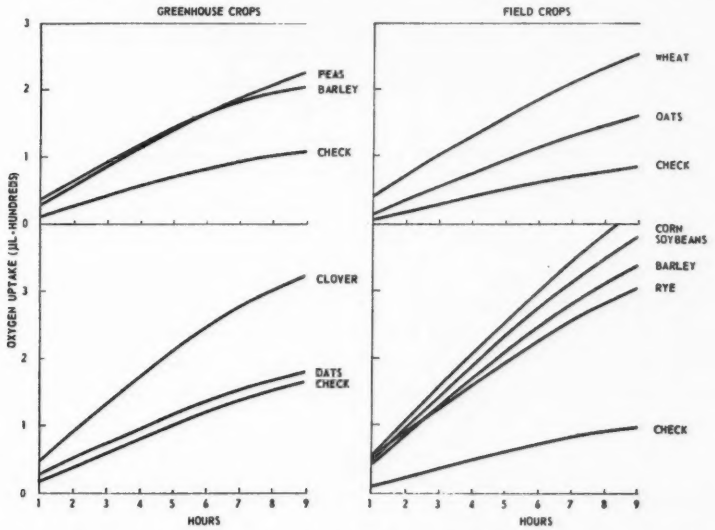

FIG. 1. Oxygen uptake by rhizosphere and non-rhizosphere soils from greenhouse and field.

dried, sprayed with 0.2% ninhydrin in 95% ethanol, and developed at 45° C. A complete set of flasks with treated and untreated soil was kept in the refrigerator as an additional control. The results (Fig. 4) show lower intensities of ninhydrin-positive spots in the refrigerated amended soil (C) than in the original sample (D). This would suggest appreciable absorption of amino acids (biologically as well as non-biologically) by the soil, or inadequate extraction. That the former alternative is the correct one was demonstrated by extracting the amino acids from soil immediately after they were added.

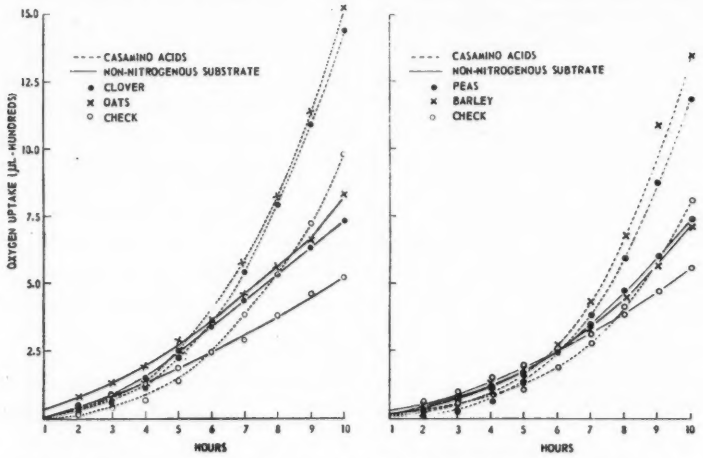

FIG. 2. Oxygen uptake by rhizosphere and non-rhizosphere greenhouse soils as influenced by nitrogenous and non-nitrogenous substrates.

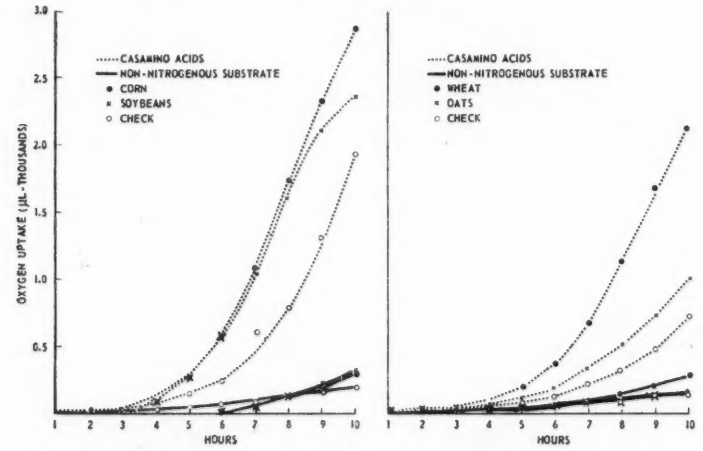

FIG. 3. Oxygen uptake by rhizosphere and non-rhizosphere field soils as affected by nitrogenous and non-nitrogenous substrates.

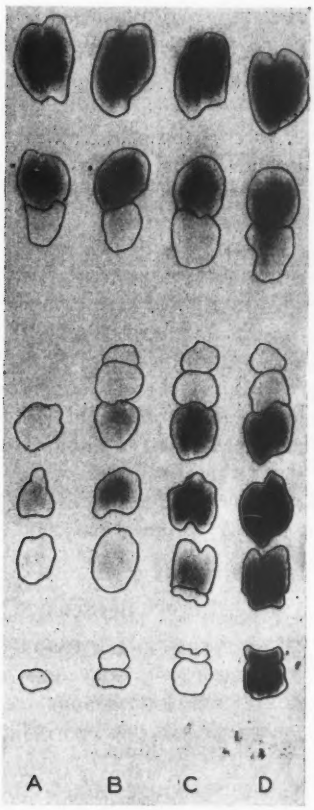

FIG. 4. Absorption and utilization of amino acids by soils. A: rhizosphere soil + casamino acids incubated for 10 hours at 30° C.; B: non-rhizosphere soil treated as in A; C: non-rhizosphere soil + casamino acids kept for 10 hours in refrigerator; D: casamino acids only; amino acids in ascending order: lysine, arginine, serine, aspartic acid, glutamic acid, alanine, proline, tyrosine, valine, phenylalanine, leucine + isoleucine.

In this case results similar to those shown in D were obtained. On incubation of the amino-acid-treated soil at 30° C. a significant decrease in intensity of the spots occurred with control soil (B) and even more so with rhizosphere soil (A). It is noteworthy that the latter yielded no spots for arginine, proline, or tyrosine; very little aspartic acid, alanine, and lysine was recovered from this soil.

Discussion

The increased microbiological activity in the soil adjacent to plant roots as recorded by various investigators (3, 5) is borne out also by the manometric studies presented above with 10 crops grown under greenhouse and field

conditions. It appears too that crop and soil differences may be demonstrated by this method which, therefore, may be a useful means of following quantitatively the over-all microbial activity in rhizosphere soil as affected by kind and age of plant and the nature and physical condition of the soil.

Recent contributions from this and other laboratories (6, 10, 12, 14) have indicated that the rhizosphere exerts a qualitative as well as a quantitative effect on the soil microflora. It has been shown also that this qualitative effect is, in part at least, in the nature of an increase in the physiological activity of the microbial constituents of this zone of root influence. As was expected, activity in terms of oxygen consumption was greater in the rhizosphere soil owing to the greater number of organisms and to the larger amount of nutrients in it than in the soil (check) a short distance away from the roots (Fig. 1). However, in addition (Figs. 2 and 3) there was an increase in the rate of oxygen uptake in the rhizosphere soils used, suggesting a physiologically more active microflora. In view of the intense competition existing in this zone it would appear reasonable to assume that the more active microbial species gain ascendancy; the results presented are not inimical to this hypothesis. Further studies with other plants at different stages of development should yield interesting information concerning these "ecological" aspects of rhizosphere investigations.

Acknowledgment

The authors are grateful to Miss M. I. Chisholm for technical assistance.

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STUDIES ON THE ISOLATION OF SPIRILLUM SPUTIGENUM[1]

J. B. MACDONALD[2] AND E. M. MADLENER

Abstract

Three approaches were used in attempting to improve the method of isolating *Spirillum sputigenum* from the human oral cavity:

(1) attempts to induce spreading surface growth;
(2) selective inhibition of interfering organisms;
(3) adjustment of oxidation–reduction potentials.

Five of eight strains of *Spirillum sputigenum* grew as spreading surface films on a blood agar medium in which the base was a veal heart infusion. Increase in the amount of spread could not be induced by varying the agar content of the medium. Sodium lauryl sulphate (0.01%) was found to have a marked inhibitory effect on the human oral flora but did not inhibit *Spirillum sputigenum*. A method of recording potentials of the surface layer of solidified media using a gold foil electrode is described. Changes in potential of the surface layer of a number of media during reduction in a Brewer jar are recorded. Media in which the potential was rapidly reduced supported growth of *Spirillum sputigenum*. Of several reducing substances added to media, sheep's serum was the most effective in accelerating a drop in potential. Using a medium compounded of veal heart infusion, sodium lauryl sulphate, and sheep's serum, *Spirillum sputigenum* was recovered in pure culture from 16 out of 21 samples of gingival scrapings.

Introduction

Spirillum sputigenum is an anaerobic member of the indigenous flora of the human oral cavity. It was observed first in 1890 by Miller (9) in scrapings from the mouth. Its presence has been reported a number of times since in mixed material from the mouth, intestinal tract, and the vagina, and it appears to have been cultivated successfully by several investigators, though most have failed to recognize it.* Two strains were described in detail by Macdonald (7), who noted that the organisms were sensitive to exposure to air and that growth occurred on blood agar only when plates were reduced immediately after streaking.

Methods of isolating this organism have included: (A) the serially inoculated shake tube method of Veillon and Zuber (23), (B) Fortner plates (22), and (C) serial streaking of blood agar. None of these methods has been found to be reliable. Macdonald (7) reported that the serially inoculated shake tube method was cumbersome compared with the serial streaking technique using Brewer jars for reduction.

Inadequacy of the isolation techniques appears to have been a major obstacle in the study of this organism. This report deals with experiments aimed at improving the method of isolating *Spirillum sputigenum*.

[1]Manuscript received February 22, 1957.
Contribution from Division of Dental Research, Faculty of Dentistry, University of Toronto, Toronto, Ontario.

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*For a review, see Macdonald (1953).

Methods

Three basic media were used: (1) Difco thioglycollate broth (B 236), (2) Difco heart infusion broth (B 38), and (3) veal heart infusion as described by Proske and Sayers (14, 15). When used as solid media the final agar concentration in all three was adjusted to 1%.

The source of material used in isolations of *Spirillum sputigenum* was gingival scrapings from human mouths, some of which had clinically-evident gingivitis or periodontal disease. Debris was collected from gingival crevices with a sterile periodontal scaler and either transferred directly to solidified media or suspended in saline or broth. Part of this crevice material was examined under the darkfield microscope, and it is worth noting that forms resembling *Spirillum sputigenum* could be observed in every case. In many attempts six strains were isolated from mixed growth in spreading surface films on blood agar media incubated in Brewer jars in a hydrogen atmosphere. These and two previously described strains (7) were used in the following experiments.

In attempting to improve the method of isolating *Spirillum sputigenum* three approaches were used: (1) attempts to induce spreading surface growth, (2) selective inhibition of interfering organisms, (3) adjustment of oxidation–reduction potential.

(1) Attempts to induce spreading surface growth: The effect of agar on the three basic media, each supplemented with 10% defibrinated sheep's blood, was studied by adjusting the concentration to 0.8, 1.0, 1.5, and 2.0%.* One plate each of the adjusted media was inoculated with three widely separated drops of a pure culture of each of eight strains of *Spirillum sputigenum*. The plates were incubated in Brewer jars in a hydrogen atmosphere for 7 days.

(2) Selective inhibition of interfering organisms: The following substances were added to Difco thioglycollate broth in various concentrations ranging between 1.0% and 0.0001%: methyl violet, brilliant green, sodium azide, sodium tellurite, indigo carmine, sodium oleate, and sodium lauryl sulphate. Growth of eight strains of *Spirillum sputigenum* in the resultant media was recorded after 7 days' anaerobic incubation. All but three of the substances proved to be inhibitory at concentrations of 0.0005% or less and were rejected for further trials. Indigo carmine, sodium lauryl sulphate, and sodium oleate were not inhibitory at concentrations of 0.1, 0.01, and 0.15% respectively.

These three substances were added in the above concentrations to veal heart infusion agar containing 10% defibrinated sheep's blood. Eight strains of *Spirillum sputigenum* were inoculated as drops as well as by the conventional streaking method on the media under test and on control plates composed of the base only. In addition, gingival scrapings from 12 sources were streaked on the test plates and on control plates without the test substances. The plates were incubated anaerobically for 7 days.

*Agar concentration influences the spread of some enteric organisms (17).

(3) Adjustment of oxidation–reduction potentials: Although all strains of *Spirillum sputigenum* grew abundantly in Difco thioglycollate broth, they failed to grow on the surface of the same medium solidified with agar. However, when 0.1% indigo carmine was added to this medium, growth occurred. As indigo carmine was presumed to serve as an oxidation–reduction compound, an investigation of the effect of potentials of solid media seemed warranted.

The salt concentration of the three basic media was adjusted to a level of 0.5% NaCl and 0.2% K_2HPO_4. A substitution was made for the thioglycollate medium: The medium used was compounded from Difco products to conform to the formula of Difco thioglycollate medium except that sodium thioglycollate and methylene blue were omitted. All media were solidified with 1.0% agar. Potentials were measured by contact between a gold foil electrode and the surface of the above three media and these same media modified by the addition of each of the following substances:

Sodium thioglycollate	0.05%
Indigo carmine	0.1 %
Defibrinated sheep's blood	10.0 %
Sheep's serum	10.0 %

The presence of agar in media impedes the diffusion of atmospheric oxygen, resulting in a decreasing gradient of potential with increasing depth of the medium (3, 24, 26, 13). It was anticipated that the agar would also impede the diffusion of hydrogen, and that therefore the more superficial layers of solidified media would be reduced more quickly than the deeper layers when the media were placed in a hydrogen atmosphere. As the reduction of the surface layer was of prime interest, the sterile electrodes and agar bridge were supported so as to touch the surface of the test medium.

The registering apparatus was a thermionic valve potentiometer (Beckman Model G) used as a zero pointing instrument. The measurements were carried out with the test media, the reference, and the measuring electrodes all enclosed in Brewer jars. Contact between the agar bridge and a half-cell was made via saturated potassium chloride solution. Wires leading from the half-cell and electrode to the registering apparatus were embedded in the plasticine seal of the jar (see Fig. 1).

The air was twice exhausted to 100 mm. of mercury and replaced each time by hydrogen. The platinized asbestos catalyzer was then heated for 30 minutes during which time atmospheric pressure was maintained inside the jar. Measurements of potential were recorded at intervals of 5 minutes for 1 hour following introduction of hydrogen into the jar and thereafter at hourly intervals for 8 hours.

Gold electrodes attain a positive potential when in contact with oxygen (6, 2). Using 1% agar plates containing salts to complete the cell and a gold electrode of standard shape and size, the change in oxygen–gold contact potential was recorded against time while the jar was being reduced according

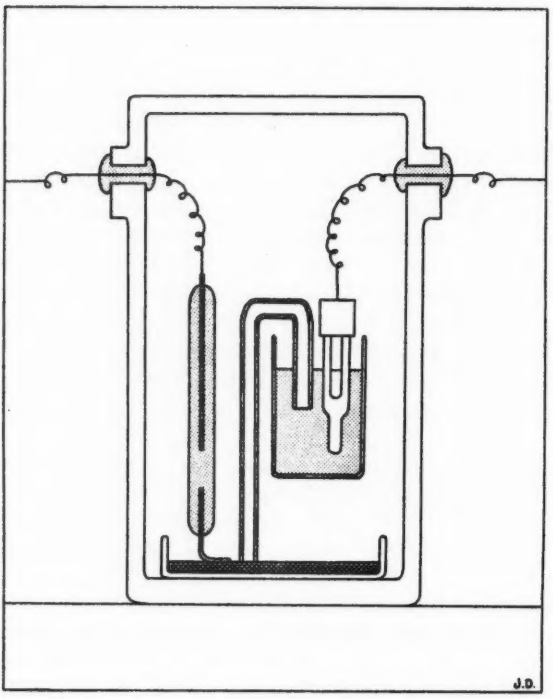

FIG. 1. Diagram of arrangement of gold foil electrode and half-cell in Brewer jar.

to the standard procedure outlined above. The resultant curve served as a base line against which changes in potential of the test media could be evaluated.

Concurrently with the measurements of potential the same media were tested for their ability to support growth of the eight strains of *Spirillum sputigenum*.

Results

Agar concentration did not influence the amount of spread of any of eight strains of *Spirillum sputigenum*. However, spreading growth was affected by the medium used. Five of the eight strains showed spreading surface growth on the media containing heart infusion base (B 38) or Proske and Sayers veal heart infusion. The widest areas of spread (up to 10 mm.) were found with the latter medium. No spreading surface growth occurred where Difco thioglycollate broth was used as a base.

All eight strains grew as well on the medium containing indigo carmine, sodium lauryl sulphate, or sodium oleate as on the control medium to which these substances were not added. Where the test media were inoculated with gingival scrapings, inhibition of part of the flora occurred. This was

most marked in the case of the medium containing sodium lauryl sulphate or sodium oleate. Inhibition on the indigo carmine plates was less marked. No isolated colonies of *Spirillum sputigenum* were observed on any of the plates. A film of spreading surface growth emanating from areas of confluent growth was noted in all cases. Darkfield examination of the films from the control plates and plates containing indigo carmine showed a variety of organisms. However, the films from the plates containing sodium lauryl sulphate or sodium oleate showed what appeared to be only two types of organisms — a motile straight rod and forms resembling *Spirillum sputigenum*. Attempts to separate *Spirillum sputigenum* from these other motile organisms by subculture failed because of overgrowth of the latter.

Changes in potential measured at the surface of various media as recorded below incorporate the changing oxygen–gold contact potential attributable to decreasing oxygen concentration in the Brewer jars. In recording the oxygen–gold contact potential against time while the Brewer jar was being reduced, it was found that the starting potential varied with the type of salt. However, the rate and extent of the potential drop during removal of oxygen was constant. In Fig. 2 measurements using two salt solutions are recorded. The extent of the potential drop was about 100 mv. in 12 minutes. This curve served as a base line in considering potential changes on the various solidified media.

The potential of all media was lowered over a period of several hours. Differences between the three basic media did not exceed 20 mv., but addition of oxidation–reduction substances influenced the extent of the drops in potential in the first hour. The greatest drop occurred when serum was added (more than 350 mv., allowing no correction for the drop attributable to the decreasing oxygen–gold contact potential). The next greatest drop occurred in media containing indigo carmine, followed by media containing blood.

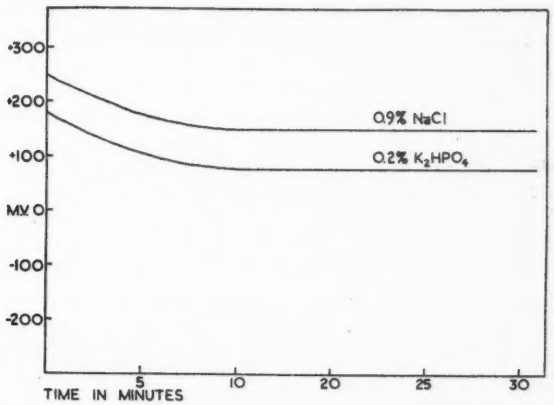

FIG. 2. Time–potential curves of the "oxygen-contact" potential of a gold foil electrode in a Brewer jar during exhausting and refilling with hydrogen.

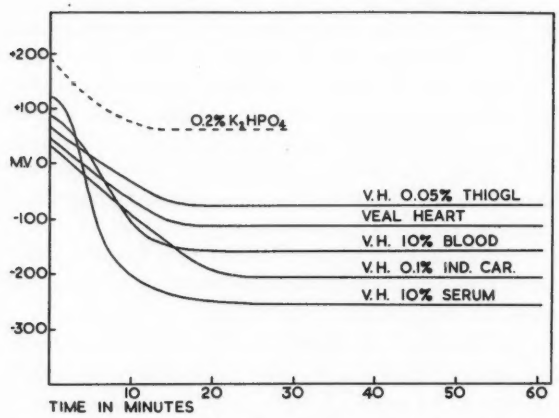

FIG. 3. Time–potential curves in a Brewer jar obtained on the surface of veal heart infusion 1% agar, and on the same medium with added oxidation–reduction substances following exhausting and refilling with hydrogen.

The potentials of the surface layer of media containing sodium thioglycollate did not differ significantly from those of the basic media without this substance. The initial rate of change in potential was greater in the presence of either serum or blood than in any of the other media. These relationships are illustrated graphically in Fig. 3. The change in oxygen–gold contact potential using 0.2% K_2HPO_4 to complete the cell is included in this figure and illustrates the extent to which apparent change in potential of the various media is due to this inherent error in the measuring procedure.

The media containing serum, blood, or indigo carmine regularly supported growth of all strains. No growth was observed on the basic media without modifiers or when sodium thioglycollate was added. These data indicate that the media supporting growth were also those in which the potential of the surface layer was lowered the greatest amount during the first hour in the Brewer jar.

Application of Results

A final series of isolation experiments was designed to take advantage of what had been learned in the various studies reported above. Proske and Sayers medium with 1% agar was used to obtain the widest possible spreading films of *Spirillum sputigenum*. Selective inhibition of part of the flora from gingival scrapings was accomplished by the use of 0.01% sodium lauryl sulphate or 0.15% sodium oleate. Ten per cent sheep's serum was used to permit rapid lowering of the potential. The plates were inoculated with drops of oral scrapings and immediately transferred to a Brewer jar. The jars were incubated for 7 days.

From the inoculated drops, films of spreading surface growth developed. The surface of the medium containing sodium oleate was finely pitted, making

observation of the texture of the films difficult. On the sodium lauryl sulphate medium two types of films were observed. One had a fine granular texture with a regular outline. The other was a coarser granular film with an irregular outline which often partly overgrew the finely granular film. Darkfield examination and subculture indicated that the film with the fine granular texture consisted of pure growth of *Spirillum sputigenum*.

In 16 out of 21 trials *Spirillum sputigenum* was successfully isolated with the above method.

Discussion

These experiments have resulted in the development of a satisfactory and reliable method of isolating *Spirillum sputigenum* from the oral cavity. Three factors appear to have contributed to the success of the method, namely, (1) encouragement of spreading growth, (2) inhibition of part of the oral flora, and (3) rapid attainment of a low potential of the surface layer of solidified media.

The third of these, i.e. potential of the surface layer of solidified media, involves an area of investigation which appears not to have been explored previously. Yet it is a common experience to have difficulty in inducing some organisms to grow on the surface of solidified media which are adequate in the fluid state (20, 25, 18, 19). As noted and reviewed by McLeod (8), there is considerable evidence to show that anaerobes in their vegetative forms die rapidly when exposed to air. McLeod suggested that death in these circumstances was due to a lethal product resulting from contact of oxygen with the organisms or some metabolite. His belief that this product was H_2O_2 has been discounted by subsequent workers except in the case of pneumococci (1, 11, 12). Furthermore, it has been shown that anaerobes will grow in the presence of oxygen provided the medium contains reduced substances such as thioglycollic acid or reduced iron (4). It has been pointed out by Wilson and Miles (27) that these findings do not eliminate the possibility that oxygen *per se* is inhibitory and that reducing substances might act to prevent its direct access to oxygen-sensitive sites. While this could well be true in a fluid medium, it seems unlikely that reducing substances could block the access of oxygen to organisms on the surface of a solid medium. Since oxygen was accessible equally to organisms on the surface of the media which would support growth of *Spirillum sputigenum* and those which would not, it seems unlikely in this case that oxygen was directly toxic. Anaerobes require a low Eh for growth to be initiated (10, 5, 21). The results in this study are not incompatible with the hypothesis that a higher Eh may be harmful.

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EFFECT OF SALT CONCENTRATION ON THE EXTRACELLULAR NUCLEIC ACIDS OF MICROCOCCUS HALODENITRIFICANS[1]

I. TAKAHASHI[2] AND N. E. GIBBONS[3]

Abstract

Labelled non-viscous cells of *Micrococcus halodenitrificans* were obtained in media containing P^{32} and 2 *M* sodium chloride and their growth then followed in salt concentrations ranging from 0.55 *M*, the lower limit of growth, to 3 *M*. At concentrations of 0.7 *M* and above, cells remained non-viscous and only traces of extracellular nucleic acids (NA) could be detected. At lower concentrations of salt, cells became viscous and the amount of extracellular NA increased as the concentration decreased. The distribution of P^{32} indicated that the source of the extracellular NA was intracellular. At the salt concentrations studied, the loss of NA was prevented by the addition of calcium or magnesium ions. However, sodium chloride seemed necessary to maintain the cell wall of *M. halodenitrificans* and the effect was supplemented by divalent ions. It is suggested that this may explain the salt requirement of this organism and possibly that of other halophilic bacteria.

Introduction

The presence of a deoxyribose nucleic acid (DNA) slime layer in *Micrococcus halodenitrificans* was reported by Smithies and Gibbons (8). The amount of DNA which could be removed from the cells with deoxyribonuclease (DNase) decreased as the concentration of salt[4] in the medium increased and it was suggested that at low salt concentrations a deficiency of essential metabolites affected the permeability of the cell and allowed the leakage of DNA or its precursors from the cell. It was also suggested that DNA slime layers might only occur in halophilic bacteria. However, Catlin (3) has reported that several non-halophilic bacteria produce DNA slimes under certain conditions and has suggested that the accumulation of DNA is the result of inhibition of DNase activity by extracellular ribose nucleic acid (RNA).

The present study was undertaken to obtain information on the source of the extracellular nucleic acids of *M. halodenitrificans*, the effect of salt concentration on production of the acids, and their relation to the halophilism of this organism.

Methods

The general procedure was to grow cells of *M. halodenitrificans* in media containing P^{32} and 2 *M* sodium chloride, a concentration at which extracellular slime is negligible and at which the cells can be readily dispersed. Cells were then transferred to isotope-free media containing various concentrations of salt and the distribution of nucleic acids in the cells, slime, and medium followed over a period in the logarithmic phase of growth.

[1]Manuscript received March 21, 1957.
Contribution from the Division of Applied Biology, National Research Laboratories, Ottawa, Canada.
Issued as N.R.C. No. 4377.
[2]National Research Council Postdoctorate Fellow 1956–57.
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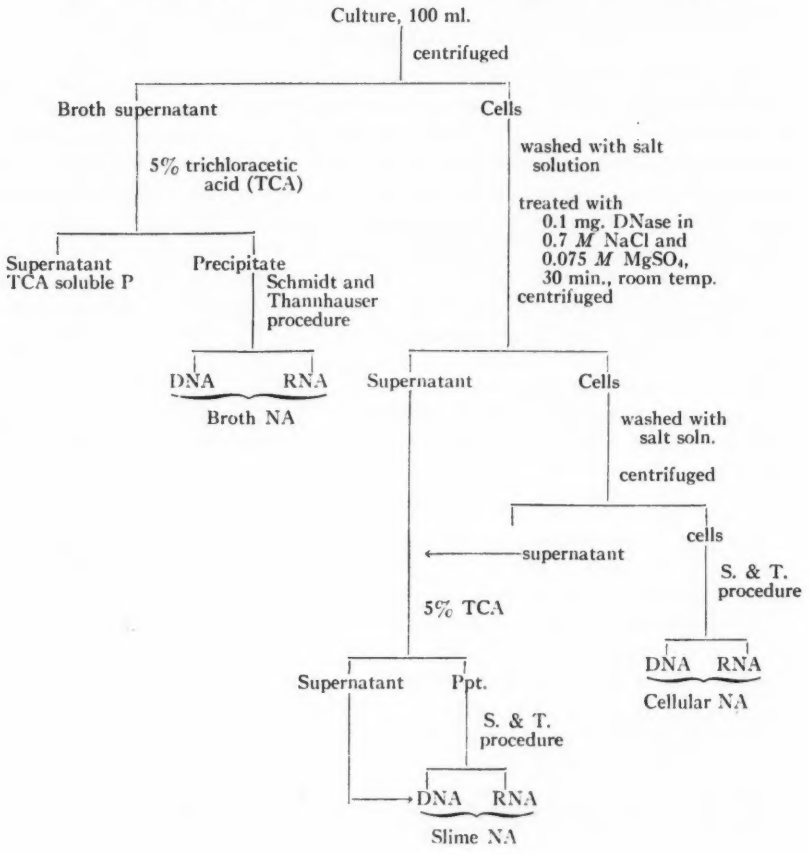
[4]Throughout this paper salt refers to sodium chloride.

The growth medium contained 0.5% each of proteose–peptone (Difco) and tryptone (Difco) and the required amount of salt (Dairy grade, Canadian Salt Co.). All cultures were incubated at room temperature (20°–25° C.) on a rotary shaker. Labelled cells were grown in broth containing 2 *M* salt, and 5 μc. carrier-free inorganic P^{32} per ml. The total phosphorus content of the medium was 52 μg. per ml. After 20 hours' incubation, the labelled cells were harvested by centrifugation and washed three times with 2 *M* salt solution. A thick suspension was then made in a sodium chloride solution of the same concentration as that of the isotope-free medium to be used. Several 250 ml. flasks, each containing 100 ml. of isotope-free medium, were inoculated with 1 ml. of the labelled suspension and incubated as above. At each sampling, the contents of two flasks were combined and analyzed.

TABLE I

METHOD OF SEPARATING NUCLEIC ACID FRACTIONS OF *M. halodenitrificans*



The nucleic acids in 100 ml. of culture were fractionated as outlined in Table I. RNA and DNA were separated by the method of Schmidt and Thannhauser as modified by Siminovitch and Graham (7). Phosphorus was estimated colorimetrically by the method of King (5). Radioactivity was determined on 0.2 ml. samples, dried on aluminum planchettes under an infrared lamp, and counted using an end-window counter and a Nuclear Model D181 scaler. DNase activity was determined by the method of Schneider and Hogeboom (6). Optical density of cultures was measured at 660 mμ with a Coleman Junior Spectrophotometer. The total cell count was determined microscopically using a Petroff-Hauser slide and the viable cell count by plating on proteose–peptone tryptone agar containing the same concentration of salt as the growth medium.

Results

Growth could not be detected in media containing less than 0.55 *M* sodium chloride after 20 hours' incubation. In the same period viscous cultures were obtained in media containing 0.55, 0.60, and 0.65 *M* salt, and the bacterial mass could not be dispersed in salt solutions. At salt concentrations of 0.7 *M* or greater, growth occurred but the cells did not become viscous, even after 12 days' incubation. In these non-viscous cultures DNase activity could not be demonstrated in broth supernatant. There was also no increase

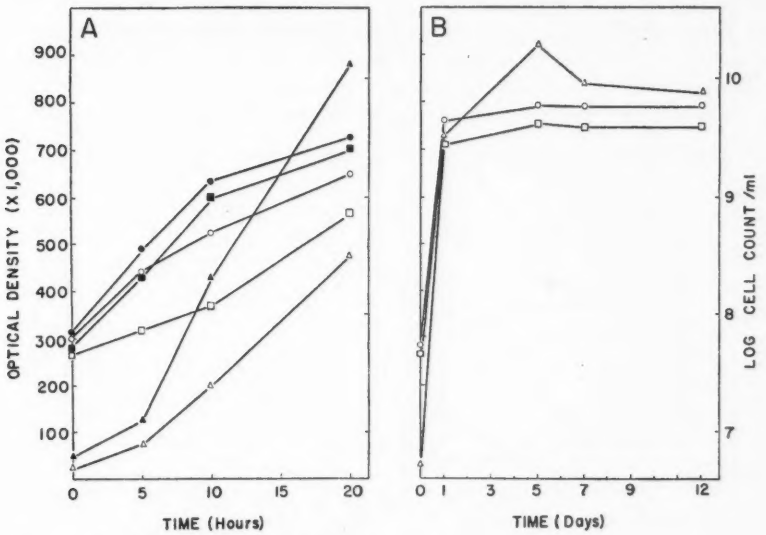


FIG. 1. Growth curves of *M. halodenitrificans*.
A: open symbols—0.6 *M* NaCl; closed symbols—1.0 *M* NaCl.
B: 0.7 *M* NaCl.
△ ▲ Optical density; ○ ● total cell count;
□ ■ viable cell count.

in substances absorbing light at 260 mμ in TCA filtrates. When 5.0×10^{-3} M. of calcium or magnesium chloride was added per liter of medium containing 0.65 *M* salt, the growth remained non-viscous indicating that divalent ions prevented slime formation. However, when calcium was not added the medium contained about 5.0×10^{-4} *M* calcium (4), and under these conditions the critical salt concentration, separating viscous from non-viscous cultures, lies between 0.65 and 0.70 *M*.

Growth curves representative of viscous cultures in 0.6 *M* salt and of non-viscous cultures in 1.0 *M* salt are given in Fig. 1A. Based on total cell count the former passed through 4.5 generations, the latter through 5.5

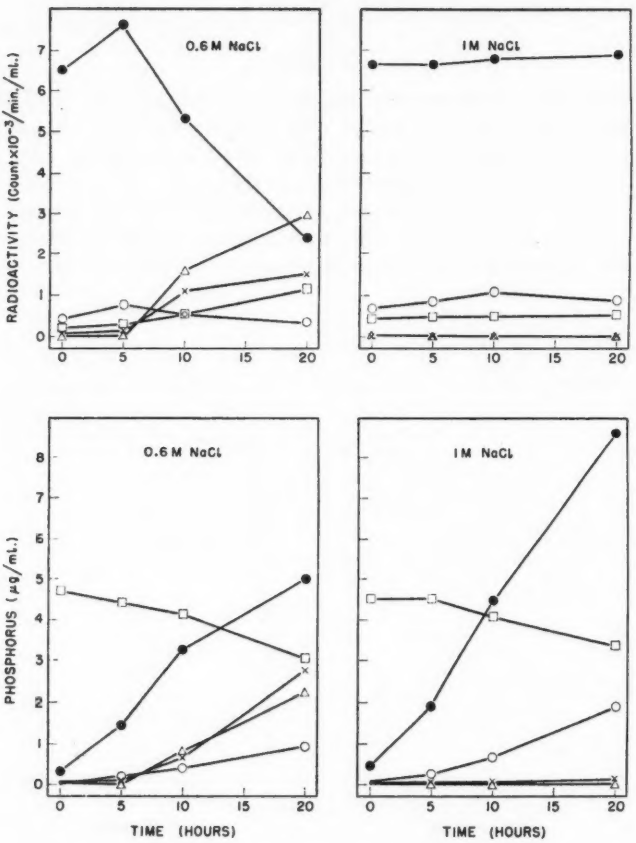


FIG. 2. Change in phosphorus content and radioactivity of nucleic acid fractions of cultures of *M. halodenitrificans* in 0.6 and 1.0 *M* sodium chloride.
● Cellular RNA; ○ cellular DNA; × slime nucleic acids; △ broth nucleic acids; □ TCA soluble P in medium (in phosphorus curves as μg./0.1 ml.).

generations in 20 hours. There was no evidence of toxicity of the isotope at the concentrations used, since growth curves of labelled cells were almost identical with those of non-labelled cells.

The changes with time in the distribution of radioactivity and of phosphorus in fractions of the same representative viscous and non-viscous cultures are presented in Fig. 2. In both cultures cellular RNA-phosphorus and DNA-phosphorus (RNAP and DNAP) increased and phosphorus compounds soluble in 5% TCA decreased. However, in 1.0 *M* salt the radioactivity of the cellular nucleic acids remained constant and practically no radioactivity or phosphorus appeared as extracellular NAP. In 0.6 *M* salt, on the other hand, the radioactivity of the intracellular NA decreased and could be accounted for almost entirely by the increase in the activity of the extracellular NA fractions. This increase in radioactivity and in NAP of the extracellular fractions became particularly evident after 10 hours' incubation when the culture became noticeably viscous. In 0.6 *M*, but not in 1.0 *M* salt, the radioactivity of the phosphorus soluble in 5% TCA increased slightly, suggesting that there was some degradation of the nucleic acids at the lower salt concentration.

Since 0.7 *M* salt seemed the lowest concentration at which extracellular nucleic acids were not found during a growth period of 20 hours, a culture in 0.7 *M* salt was followed over a period of 12 days (Figs. 1B and 3). Although there was a barely perceptible increase in the extracellular NAP during this time, there was a slight increase in the radioactivity of the broth nucleic acid fraction for the first day and a steady increase in that of the TCA soluble fraction throughout the experiment. Since there was practically no change in the radioactivity of the cellular DNA fraction, it is assumed that most of this

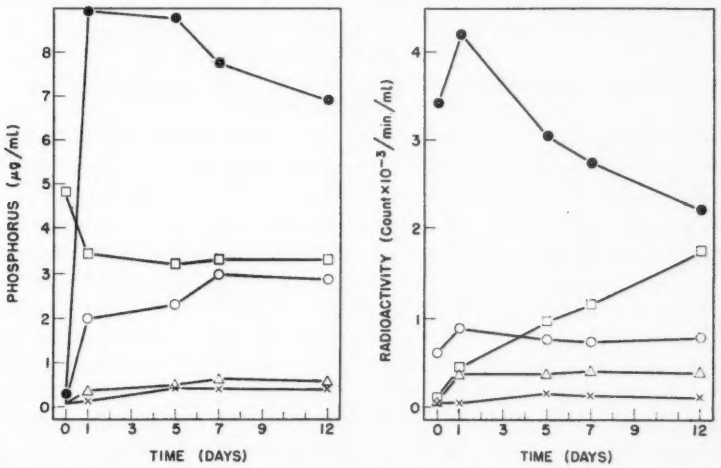


FIG. 3. Change in phosphorus content and radioactivity of nucleic acid fractions of a culture of *M. halodenitrificans* in 0.7 *M* sodium chloride. Symbols as in FIG. 2.

increase was at the expense of the cellular RNA fraction, the activity of which decreased steadily after the 1st day. This is probably similar to the loss of cellular RNA noted by others in aging cultures of *Escherichia coli* (1) and of *Bacterium lactis aerogenes* (2).

At the end of 20 hours' incubation the NAP content per cell was nearly constant (3.1–4.2 $\times$ 10^{-9} µg.) regardless of the salt concentration of the medium (Table II). At concentrations of 0.7 *M* salt and greater, the extracellular NAP per cell was very small. However, at 0.65 *M* salt about a third of the total NAP was extracellular and at 0.55 *M*, the lowest concentration at which growth would occur under the conditions of these experiments, three-quarters of the NAP was extracellular. The percentage decrease in radioactivity of cellular NA at these lower concentrations agreed very well with the percentage increase of extracellular NAP.

TABLE II

CELLULAR AND EXTRACELLULAR NUCLEIC ACIDS IN 20 HOUR CULTURES OF *M. halodenitrificans* GROWN AT DIFFERENT SALT CONCENTRATIONS

Concentration NaCl, *M*	Optical density	Cellular NAP/cell, 10^{-9} µg.	Extracellular NAP/cell, 10^{-9} µg.	Extracellular NAP in total NAP, %	Decrease in radioactivity of cellular NA, %*
0.55	0.090	3.3	11.4	78	81
0.60	0.475	3.6	3.1	46	60
0.65	0.440	4.2	2.2	34	43
0.70	0.490	3.5	0.3	8	1
0.85	0.515	3.4	0.2	6	0
1.0	0.880	3.3	0.0	0	0
2.0	0.575	3.5	0.2	5	0
3.0	0.385	3.6	0.1	3	0
0.65 + Mg†	0.630	3.1	0.1	3	0
0.65 + Ca†	0.600	3.2	0.1	3	0

* $\frac{\text{Initial radioactivity} - \text{radioactivity after 20 hours}}{\text{Initial radioactivity}} \times 100$.

† 0.005 *M* $MgCl_2$ or $CaCl_2$.

TABLE III

DISTRIBUTION OF RNA AND DNA IN FRACTIONS OF *M. halodenitrificans* AFTER 20 HOURS' GROWTH

Fraction	Conc. NaCl, *M*	RNAP/100 ml. culture (A), µg.	RNAP/cell,* 10^{-9} µg.	DNAP/100 ml. culture (B), µg.	DNAP/cell,* 10^{-9} µg.	Ratio $\frac{\text{RNAP (A)}}{\text{DNAP (B)}}$
Cells	0.55	92	2.9	15	0.5	6.1
	0.60	497	3.1	91	0.6	5.4
	0.65	459	3.5	94	0.7	4.8
	0.70	610	2.9	135	0.6	4.5
	0.85	623	2.8	134	0.6	4.6
	1.00	863	2.7	188	0.6	4.5
	2.00	535	2.9	116	0.6	4.6
Slime	0.55	25	0.7	49	1.5	0.5
	0.60	106	0.7	169	1.0	0.6
	0.65	36	0.3	74	0.6	0.5
Broth	0.55	257	8.0	35	1.1	7.3
	0.60	192	1.2	33	0.2	5.8
	0.65	143	1.1	26	0.2	5.5
Total extracellular	0.55	282	9.0	84	2.6	3.4
	0.60	298	1.8	202	1.2	1.5
	0.65	179	1.4	100	0.8	1.8

*Based on total cell count.

Although it was mentioned in a previous paper (8) that some RNA was present in the slime layer, no quantitative data were given. The data presented in Table III show that there is approximately twice as much DNAP as RNAP in the slime layer and five to seven times as much RNAP as DNAP in the medium. Data are also presented indicating that the ratio of RNAP to DNAP is constant in non-sticky cells. As the salt concentration is lowered and the cells become more sticky this ratio increases in all fractions.

Quantitative data are given in Table II which support an observation made earlier (8) that small amounts of calcium added to the medium prevent the loss of nucleic acids from the cell. The amount of calcium added in the present experiments was about ten times, and of magnesium 100 times, the amount present in the usual medium (4). Usually, in media containing 0.7 *M* salt the concentration of calcium was 0.3×10^{-4} *M* greater, and of magnesium 0.2×10^{-5} *M* greater, than in media containing 0.65 *M* salt. This slight difference in the concentration of calcium and magnesium is hardly sufficient to account for the appearance of extracellular nucleic acids. The occurrence of extracellular nucleic acid, therefore, is probably dependent on the concentration of sodium chloride.

Discussion

The accumulation of extracellular DNA in non-halophilic bacteria under conditions which minimize DNase activity (presence of 1 *M* NaCl or pH 6) has been reported by Catlin (3), and attributed to the inhibition of DNase by extracellular RNA. This explanation implies that extracellular nucleic acids are produced by these organisms under all conditions, but that normally they are depolymerized by extracellular enzymes. However, in cultures of *M. halodenitrificans* in 1 *M* sodium chloride, growth is maximal, extracellular nucleic acids are minimal, and DNase activity cannot be detected in broth supernatants. The mechanism of extracellular NA accumulation in cultures of *M. halodenitrificans*, therefore, seems different from that in cultures of the non-halophilic bacteria studied by Catlin.

It is evident, from the distribution of radioactivity in cultures grown at low salt concentrations, that the source of the extracellular NA is intracellular NA. The nucleic acids must, therefore, have passed through the cell membranes, or been released from ruptured cells. The first assumption would necessitate very decided changes in the permeability of the cell membranes to allow the highly polymerized DNA found extracellularly to pass through. It would also mean that a cell growing in 0.55 *M* salt would have to synthesize four times as much NA as one growing in 1.0 *M* salt to maintain the constant level of intracellular NA found. Thus a change in the permeability of the cell membrane does not offer a satisfactory explanation of extracellular NA.

A more reasonable explanation is that between 0.7 and 0.5 *M* salt there is a transition from normal viable cells to non-viable cells, some at least of which rupture and release NA into the medium. Studies now in progress indicate that in the intermediate concentrations normal viable cells occur, but the

proportion of these decreases with the salt concentration while the number of swollen and broken cells increases. The ruptured cells release cytoplasmic and nuclear components into the medium; most of the DNA adheres to the cells and the RNA diffuses into the medium. Even the ruptured cells do not disintegrate completely; otherwise the ratio of RNA to DNA in the extracellular fractions would be the same as in the cellular fractions. The ratio in the extracellular fractions tends to approach that of the cellular fractions as the salt concentration decreases, indicating a more complete breakdown of the ruptured cells. At the same time, the proportion of DNA in the cellular fraction decreases, which probably means that the DNase used to remove the extracellular slime from the cells (Table I) is also able to remove more DNA from the cell fragments, although these are still large enough to sediment with the whole cells.

Salt, therefore, seems necessary to maintain the cell wall of *M. halodenitrificans*. In the presence of the very low concentrations of calcium and magnesium provided by the medium, a concentration of at least 0.7 *M* sodium chloride is required. However, small additions of calcium, and to a lesser extent magnesium, help the organism to maintain the cell wall and allow it to survive and even grow at salt concentrations less than 0.55 *M*, the usual lower limit of growth. It has been noted (unpublished results) that in the presence of 0.5×10^{-3} *M* calcium chloride, the growth range of *M. halodenitrificans* can be extended to 0.3 *M* salt and that the cells were not viscous. However, below 0.3 *M* salt, growth was negligible even in the presence of 0.01 *M* calcium or magnesium. Calcium, therefore, supplements the salt requirement but does not replace it. This need of sodium chloride to maintain the cell wall probably explains the minimum salt requirement of *M. halodenitrificans* and possibly that of other halophiles.

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DISEASE OF THE LARVAE OF TENT CATERPILLARS CAUSED BY A SPOREFORMING BACTERIUM[1]

G. E. BUCHER[2]

Abstract

A newly discovered bacterial disease of larvae of *Malacosoma pluviale* (Dyar) (Lep.) is caused by infection of the gut with a large, motile, sporeforming bacterium, *Bacillus* sp., that increases in size before sporulation and bears the spore without bulging. It has not been cultivated. The bacterium invades the host with the food and multiplies in the midgut and foregut, producing changes in the pH and causing dysentery in the host. Sporulation occurs in the gut and both rods and spores are passed in the faeces and spread the disease. The infected larva loses its appetite, regurgitates excessively, produces wet faeces, decreases markedly in length, and dies in a characteristic, short, dry, mummified condition after about a week. Small ingested doses of spores initiate infection in laboratory populations of *M. pluviale* in all instars. *M. americana* (F.) also is susceptible to the disease but *M. disstria* Hbn. is resistant, only a few individuals dying from it.

Introduction

In 1954, difficulty was experienced in rearing some populations of the western tent caterpillar, *Malacosoma pluviale* (Dyar), collected near Vancouver, B.C. The larvae ceased to feed, progressively shortened in length, and died before pupation in a short, mummified condition suggestive of the effects of starvation. Preliminary examination of the moribund and dead larvae revealed a small number of bacterial spores in the gut and excreta. A quantity of spore material was collected and used in 1955 to initiate the disease in healthy larvae. This paper is an account of observations and experiments made in 1955 on *M. pluviale* and on the eastern tent caterpillar, *M. americanum* (F.), and the forest tent caterpillar, *M. disstria* Hbn. Unless otherwise noted, statements refer to the disease in *M. pluviale*.

Materials and Methods

Larvae of *M. pluviale* were collected in the area of Vancouver, B.C., those of *M. americanum* near Kingston, Ont., and those of *M. disstria* in both areas. Larvae were reared in lots of 25 in small dishes lined with paper toweling, or singly in 20 × 70 mm. tubes, and supplied with one or two changes of fresh food daily as required. *M. pluviale* was fed on leaves of apple (*Malus* sp.), *M. disstria* on those of willow (*Salix babylonica* L.), and *M. americanum* on those of eastern choke cherry (*Prunus virginiana* L.). To minimize cross contamination by disease organisms, lots were formed of individuals from a single web, and a record of the web of origin was maintained. In some webs, insect parasitism and virus or bacterial infection were sufficiently high to interfere with experiments, and insects from them were discarded. The temperature fluctuated between 20° and 25° C.

[1]Manuscript received February 21, 1957.
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The larvae were fed known doses of the bacterial spores by confining each in a vial with a small piece of leaf on which a 0.01 ml. drop of spore suspension had been allowed to dry. They usually consumed the entire segment of leaf within 1 hour. After they had been fed, the larvae were reassembled in lots of 25 or maintained singly in vials. The concentration of the spore suspension was estimated microscopically with a bacterial counting chamber.

The spores were collected from the anal discharge of sick larvae and were contaminated with other organisms. Three spore suspensions were tested to determine the efficiency of purification and the resistance of the spores to the purification treatments. Suspensions cleaned by repeated washing and centrifuging were called raw. Pasteurized suspensions were raw suspensions that had been heated at 60° C. for 90 minutes. Chemical suspensions were raw suspensions that had been treated with phosphate buffer for 2 hours at pH 11 to minimize the occurrence of active virus. Concentrated raw and chemical suspensions contained a small number of viable bacteria and yeasts; pasteurized suspensions were sterile for yeasts and non-sporulating bacteria. Polyhedra were not visible in any suspensions. As all three suspensions were equally effective in producing the disease and as the mortality from other causes was not reduced by pasteurization or chemical treatment, the results have been averaged (Tables I–IV).

The development of the infection in individual larvae was followed for several days by smearing the regurgitated drops and the faeces. These observations were supplemented by dissecting and smearing portions of the gut of selected larvae at intervals after ingestion of an infective dose. Microscopic examination was made immediately on wet smears with a dark-field condenser. Dark-field preparations were not suitable for photography because the bacteria were motile and the spores much more refractive than the vegetative rods. Permanent stained slides were prepared by fixing the partially dried smears with Carnoy's solution for 2 minutes, staining for spores with Ziehl's carbol fuchsin in a boiling water bath for 5 minutes, and decolorizing them in 95% ethyl alcohol. The vegetative portions of the rods were counterstained with Giemsa* by a method used by E. U. Canning (in litt.), University of London, England, for microsporidia. The smears were placed in freshly made Giemsa stain for 2 to 5 minutes, decolorized rapidly in colophonium–acetone, washed in acetone, transferred through two acetone–xylol mixtures to xylol for clearing, and mounted in neutral mounting medium. After this treatment, the spores stained red and the vegetative rods stained degrees of blue. This

*Composition of Giemsa stain:

Giemsa stock stain	10 ml.
Acetone	10 ml.
Methyl alcohol	10 ml.
Sørensen buffer, pH 7.2 diluted 1/20	100 ml.

Composition of differentiating fluid:

Colophonium resin	15 g.
Acetone	100 ml.

The stock stain was prepared with one parstain vial of Giemsa, as directed by the manufacturer, Hartman-Leddon Co., Philadelphia.

procedure had the advantage over others of not strongly staining the slime and small particles of debris, so that bacteria stood out sharply against a clear background; as the smears were not dried, the bacteria underwent less shrinkage and distortion than in conventional staining methods.

External Symptoms of Infected Larvae

The visible external symptoms of the disease in the larvae varied in degree and particularly in the time at which they appeared. Factors affecting the onset of symptoms were chiefly individual resistance of the larvae, secondary infections, and, to a lesser extent, the size of the dose, the age of the larva, and the temperature. Thus the times mentioned below are modal estimates.

Larvae showed no change during the 1st 24 hours after ingestion of the spores. During the 2nd day, larvae became more irritable and regurgitated readily when stimulated; healthy larvae did not regurgitate except under strong, repeated stimulation. During the 3rd day, larvae showed clear symptoms; they ate less and voided wet faeces that made characteristic rusty-brown stains on the substrate. At the same time they showed a diagnostic shortening and as they shrank in length they became increasingly sluggish and less responsive to stimuli. All symptoms became progressively more strongly marked until the larvae became moribund on the 5th or 6th day. Before reaching the moribund stage, the larvae emptied most of the gut contents and excreted a dense, rusty-brown fluid that contained large numbers of bacterial spores and little undigested plant tissue. Moribund larvae were short and dry, showed paralysis of the abdomen, and reacted to strong stimuli only by weak motions of the thorax. Larvae died in 1 to 4 days after becoming moribund. Dead larvae were dry, mummified, resistant to putrefaction, and very short; in extreme cases they were only a quarter as long as healthy larvae of the same instar (Fig. 1).

The Causal Agent

The causal agent of the disease is a sporeforming bacterium, apparently hitherto undescribed, and hereby assigned to the genus *Bacillus* as *Bacillus* sp. until it can be isolated, grown in pure culture, and properly characterized. All attempts to cultivate it on a wide variety of laboratory media under both aerobic and anaerobic conditions failed. Media designed to cultivate *Bacillus larvae* White and *Bacillus popilliae* Dutky did not support growth. The description that follows pertains only to its appearance within the gut of the host.

The bacteria exist as single rods, never in chains. They multiply by plate formation and division, and separate soon after division so that never more than two are associated. Both vegetative and sporulating forms display an extremely wide range in size. The size is best shown in the dark-field where a bright line is visible surrounding the cell (Fig. 3). When suspended in the gut juice of the insect and examined by dark-field, the vegetative rods were 3 to 13 μ in length by 0.9 to 1.3 μ in diameter: the majority were 6 to 7 μ in

length and about 1.0 μ in diameter; sporulating rods were 7 to 14 μ in length by 1.4 to 2.0 μ in diameter; the majority were 9 to 10 μ in length and 1.5 to 1.7 μ in diameter. Rarely, sporulating rods 17 to 20 μ long occurred. Fixing and staining caused an apparent reduction in size. After fixation with Carnoy's fixative, staining for spores, and counterstaining with Giemsa, most vegetative rods, as measured from photographs, were 5 to 6 μ in length by about 0.7 μ in diameter and most sporulating rods were 6 to 8 μ in length by 1.2 to 1.3 μ in width. The reduction in size is apparently caused by shrinkage of the cytoplasm and failure of the cell wall to stain, and is most marked when bacteria have been allowed to dry in smears before staining.

The bacteria are typically straight rods with parallel sides and rounded ends, but sometimes they are slightly curved or, when very long, have a tendency toward a gentle "S" curve (Figs. 3, 5).

The vegetative rods are optically empty and apparently free of inclusions when examined by dark-field. In sporulating rods, the spore or the developing forespore is highly refractive and appears in brilliant contrast to the remainder of the rod; sometimes there is a condensation of material with light-scattering properties along the cytoplasmic membrane. Crystalline inclusions, as reported by Hannay (4) for *Bacillus thuringiensis* Berliner and by Angus (1) for *B. sotto* Ishiwata, were not seen in the sporulating rods, either in dark-field or in air-mounted nigrosin films.

The bacteria stain readily with the usual bacterial stains. They stained Gram-negatively by various modifications of the Gram stain, even when young and actively dividing. When lightly stained with Giemsa by the technique described above, the vegetative rods colored unevenly and had a vacuolar appearance (Fig. 8). This was especially prominent in rods nearing maturity or rods that showed some development of the forespore; young, dividing rods usually stained more uniformly. Sporulating rods also stained unevenly with Giemsa.

The bacteria, both vegetative and sporulating rods, are highly motile in the gut juices of the host. However, not all of the cells of a single preparation were motile and in some preparations only rare cells were motile. Preliminary observations indicated that motility was an attribute of free-living cells in the fore- and mid-gut of the host during the period of most rapid multiplication,

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FIG. 1. Healthy and infected *M. pluviale* larvae in the fifth instar. Infected larvae show diagnostic shortening; the two on the left are dead, the two on the right moribund.

FIG. 2 (left center). Diagrammatic representation of stages in development of *Bacillus* sp.; across the top, small young rods; left center three vegetative rods, the lower two being mature; right center, two rods showing stages in spore formation; lower left, two mature sporangia with large oval spores; lower right, a chain of three sporulating rods of *Bacillus cereus* to show relative size.

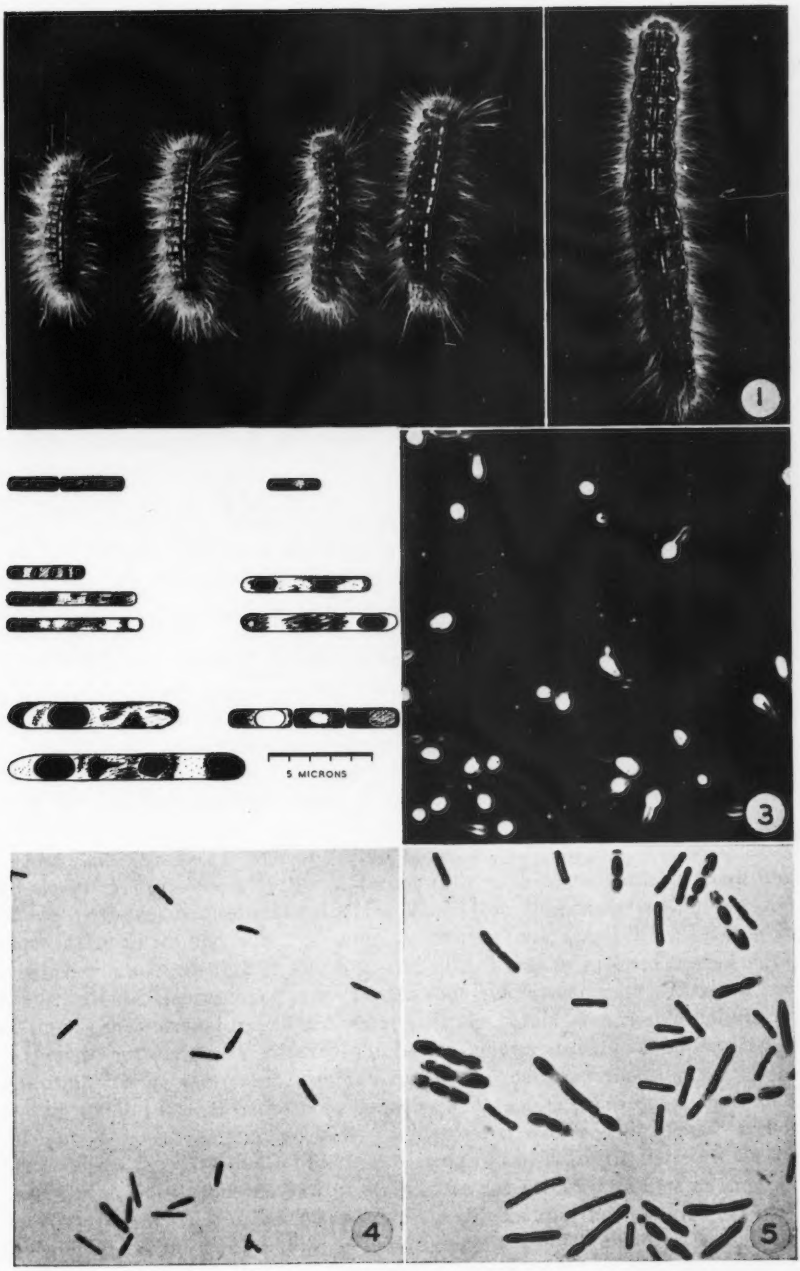
FIG. 3. Dark-field photograph of living *Bacillus* sp. in regurgitation of infected larva (×780). The bright spots are spores whose high refractivity makes them appear larger than the rods and out of focus.

FIGS. 4, 5. Stained smears of *Bacillus* sp. from the gut of *M. pluviale* at various stages of infection (×1610).

FIG. 4. Regurgitation, 16–24 hours after infection, showing young vegetative rods.

FIG. 5. Midgut, 24–36 hours after infection, showing bacteria in all stages.

PLATE I



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about 18–48 hours after infection. The pH of the gut juices may be important in determining motility. Cells growing in dense masses along the wall of the midgut showed little or no motility. Attempts to stain the flagella by the methods of Leifson (5) and Bailey (Conn and Darrow (3)) failed; the gut fluids seriously interfered with the staining reactions, and attempts to wash the bacteria free of these fluids apparently destroyed the flagella.

Mature spores of the bacteria, within the sporangium or freshly liberated, were about 1.7 μ in diameter and 3.0 to 3.5 μ in length in the dark-field. They appeared smaller, about 1.2 μ in diameter and 2.2 μ in length, when stained by carbol fuchsin. Spores were resistant to ordinary stains but young spores stained readily with hot carbol fuchsin and retained the red color when treated with alcohol. Older spores, free in the anal discharge, were more refractory to the stain and also more readily decolorized, and frequently showed up as red central areas surrounded by a colorless halo (Fig. 15). The presence of the spore did not cause any swelling of the sporangium. On the contrary, in many stained specimens, the sporangium was slightly constricted in the vicinity of the spore. This phenomenon was not visible in dark-field preparations, and is presumably an artifact caused by the absence of readily stained material immediately surrounding the spore. Typically, spores are borne in a subcentral position in the sporulating rod, but occur in almost every position except the truly terminal one. In long rods (12–14 μ), the spore occupies a subterminal position as if the rod were a pair that had failed to divide completely and only one member had produced a spore. In the dark-field some of these long sporulating rods show a bright line bisecting the rod off center away from the spore and suggesting a partial septum. Division is apparently halted at this stage, before a cell wall is completed or separation occurs, and only one member of the double rod lengthens and forms a spore. In the rare cases in which rods are 18–20 μ long and bear the spore in a nearly terminal position, complete division has apparently failed twice and the rod may be considered as triplex.

The bacterium has a cycle of development, the various stages of which may be shown at any one time by different cells within the same preparation. These are shown diagrammatically (Fig. 2). (Note that the following measurements refer to stained cells.) Young vegetative rods, about 2.5 μ long, grow until they reach a length of 5 to 6 μ, and then divide or proceed to sporulate. If they divide, the resulting pair of small rods quickly separate. Rods of two or three times normal length are apparently the result of incomplete division. If they do not divide, they increase in both diameter and length and stain less uniformly than before. When they reach the mature dimensions of about 6–7 $\mu \times$ 0.9–1.0 μ, the forespore becomes visible as a small dense mass in a subcentral to subterminal position; it stains red with carbol fuchsin and is refractive in the dark-field. The sporulating rod increases in diameter as the spore develops, so that the mature spore does not produce a bulge in the rod. Shortly after maturation of the spore, the rod lyses and the spore is liberated. Germination of the spore was not seen with certainty. The factors determining whether a rod divides or sporulates are not known.

As the bacterium was not grown in pure culture, the various forms might represent different species rather than developmental phases of the same species. The following observations support the view that they comprise a single species: the rods can be arranged in a continuous series according to size and only the small rods show evidence of division; all forms may be highly motile, all stain Gram-negatively, and all have a vacuolar appearance when lightly stained with Giemsa; time-sequence studies on artificially infected insects showed that the population as a whole follows the pattern of development described for a single organism; nutrient plates seeded with gut juices containing heavy suspensions of the bacteria remained sterile; it is unlikely that contaminating organisms would be also highly fastidious.

The Course of the Infection in the Gut

The course of the infection in the gut was followed on experimentally infected larvae. In the healthy insect the gut is relatively free of micro-organisms; smears of the fore- and mid-gut appeared to be sterile when examined microscopically, and culture media inoculated with this material remained sterile or showed the presence of rare organisms; the hindgut frequently contains bacteria and yeasts in small numbers, evident in smears or by culturing.

During the first 16 to 24 hours after ingestion of the spores, vegetative rods appear in the gut (Fig. 4). They are most numerous in the anterior portion of the midgut but occur in numbers throughout the foregut and midgut, and, to a lesser extent, in the hindgut. A few rods begin to sporulate towards the end of the period.

During the next 12 hours the bacteria multiply rapidly. The heaviest concentration occurs in the anterior half of the midgut but the bacteria are numerous in the regurgitation fluid, the foregut, and all of the midgut, and less numerous in the hindgut. The bacteria occur in all stages of their life cycle (Fig. 5). Numerous pairs can be seen in various stages of division; most of the rods are in the vegetative stage but many show some development of the forespore and a smaller number bear mature spores; a small number of free spores may be present, especially in the hindgut.

In the lumen of the gut, rods appear to be strongly attracted to cells of the insect's food and arrange themselves in radial fashion about certain leaf cells like pins in a pincushion. The "pincushion" effect is characteristic of the organism (Fig. 6); it is most evident in the anterior half of the midgut, 24 to 48 hours after infection, but sometimes occurs in the posterior half of the midgut or rarely in the foregut.

FIGS. 6–11. Stained smears of *Bacillus* sp. from the gut of *M. pluviale* at various stages of infection (×1610).

FIG. 6. Leaf cell in midgut, surrounded by bacteria showing the "pincushion" effect, 24–36 hours after infection.

FIGS. 7–10. Regurgitation, foregut, anterior midgut, and posterior midgut respectively of the same larva, 36–48 hours after infection. Note the concentration of rods in the foregut and anterior midgut, and the vacuolated appearance of some rods.

FIG. 11. Midgut, 48 hours after infection, showing heavy sporulation.

PLATE II

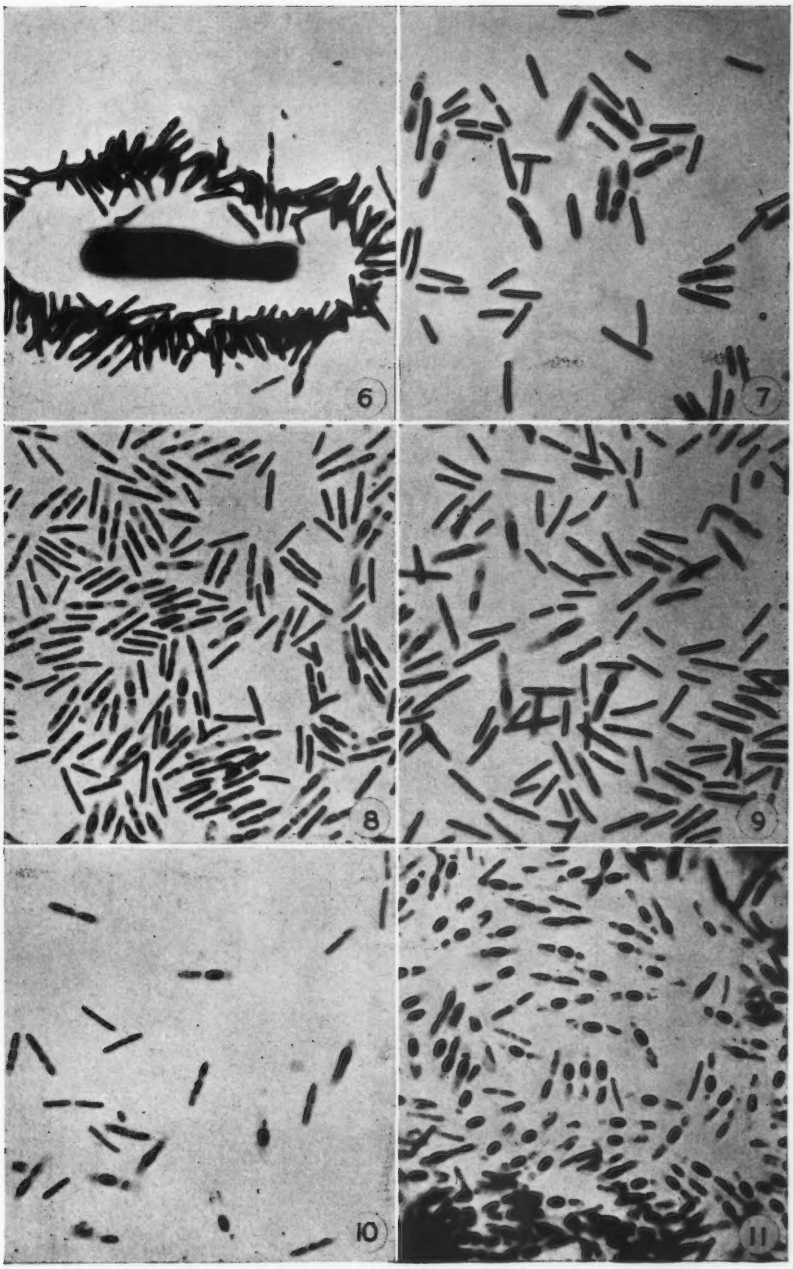

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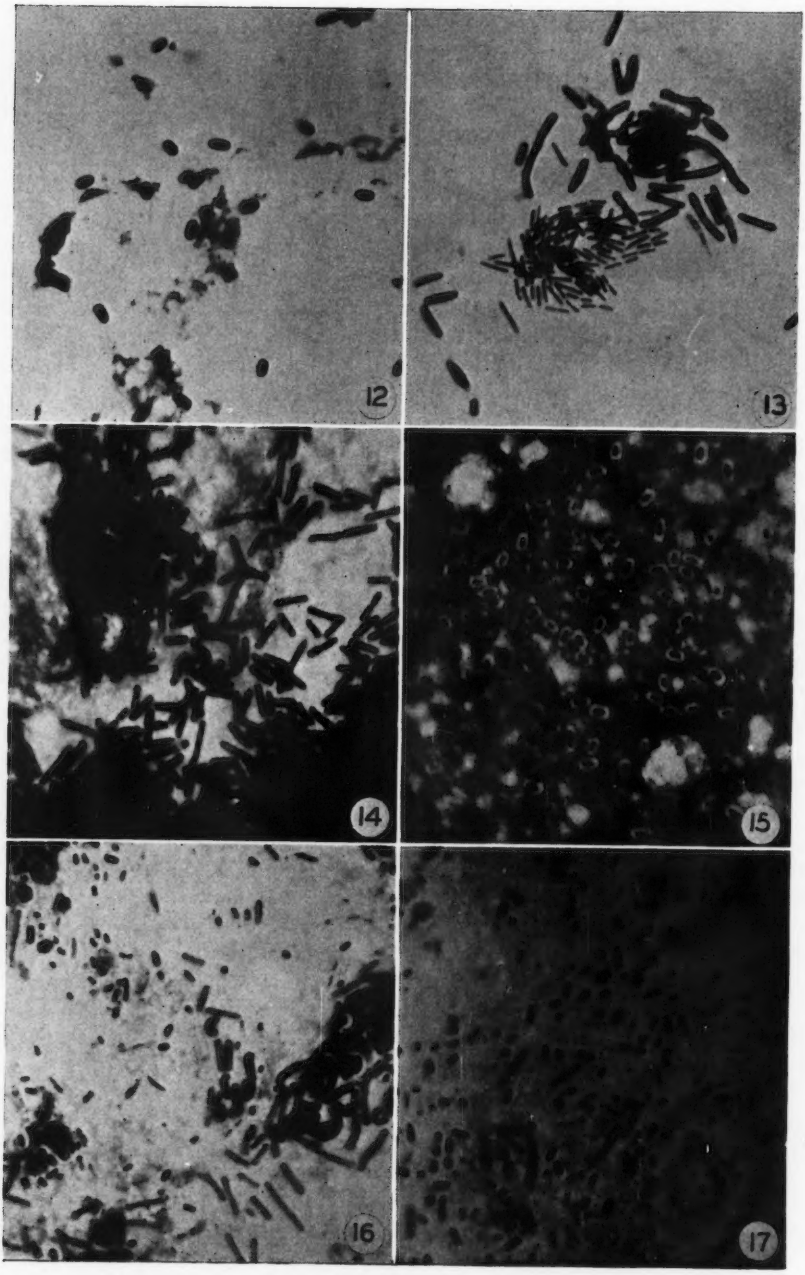
PLATE III



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In the 36 to 48 hour period the infection is at its height and definite external symptoms of infection become evident. The bacteria are most numerous in the central portion of the midgut but all parts of the gut are heavily infected (Figs. 7–10). Towards the end of the period many of the rods that are free in the lumen of the gut bear mature spores (Fig. 11). Free spores are common in the hindgut (Fig. 12) and in the frass, and a smaller number occur in other portions of the gut.

During the 3rd day of infection, variations in behavior are more pronounced. In some hosts, sporulation is completed and the gut contains numerous spores and only a small number of vegetative or sporulating rods. Spores are numerous in the faecal discharge. In other hosts secondary infections by other bacteria may develop (Fig. 13); they are most pronounced in the mid- and hind-gut and culture media inoculated with portions of the gut contents may show the growth of a variety of bacteria and yeasts.

During the 1st 2 days of infection, many bacteria are free in the lumen of the gut, but also many are applied closely to the striated border of the epithelial cells of the midgut or occur in small masses in folds between the epithelial cells. On the 3rd day, as the host begins to shorten, the longitudinal muscles of the midgut also contract, shortening the gut and producing extensive folding of the epithelial cells. Large masses of non-motile, vegetative rods, often in active division, occur in these folds, protrude into the lumen, and are sometimes detached (Fig. 14). Such masses of bacteria occur most frequently in the posterior portion of the midgut, which has an abnormal, translucent, yellowish appearance under the dissecting microscope. The bacteria in these masses may not sporulate at the same time as the other free-floating bacteria in the gut. Thus it is common, on the 3rd day, to find great masses of vegetative rods in the midgut, and, in the hindgut and faeces, large numbers of spores (Fig. 15) produced by the free-living bacteria of the fore- and mid-gut. These spores are refractory to stain and appear smaller than spores that occur within the sporangia or that are freshly discharged.

During the 4th day, when larvae are greatly shortened and desiccated, they regurgitate less readily and the foregut and regurgitation fluid may be almost free of primary infection, and may or may not show infection by secondary bacteria or yeasts. In the midgut, sporulation occurs among the rods that make up the masses of bacteria mentioned above, and secondary infection may or may not be considerable. Shortening of the midgut, a process that became apparent on the 3rd day, is more pronounced and the host begins to

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FIGS. 12–17. Stained smears of *Bacillus* sp. from the gut of *M. pluviale* at various stages of infection (×1610).

FIG. 12. Hindgut, 48 hours after infection, showing readily stained spores in the debris.

FIG. 13. Secondary bacterial infection in midgut after 3 days.

FIG. 14. Portion of large mass of vegetative rods in posterior midgut after 3 days.

FIG. 15. Spores in hindgut, 3 to 4 days after infection; they are refractory to the stain which is decolorized from the periphery; this produces a halo effect when spores are mixed with debris.

FIGS. 16, 17. Anterior and posterior midgut contents of same moribund larva showing poorly stained *Bacillus* sp. undergoing lysis and heavy infection with secondary bacteria.

empty the gut and voids very fluid faeces composed largely of cellular debris of the epithelial cells of the midgut, large numbers of spores and some rods, and, to a lesser extent, remains of leaf particles. Secondary infections by both bacteria and yeasts commonly develop in the hindgut, and numbers of these organisms are also voided with the faeces. These processes continue until the host becomes moribund on the 5th or 6th day.

There is little evidence of the primary infection after the larva has become moribund. Secondary infections by bacteria and yeasts are then rampant in all parts of the gut. In the midgut (Figs. 16, 17), there are numbers of large vegetative rods that are the remnants of the large masses of bacteria present on the 3rd and 4th days. These rods stain poorly, appear to be in various stages of autolysis, and are probably dead. Secondary bacteria and yeasts are very numerous in the hindgut together with a few ghosts of the primary rods and a few spores. A similar picture is obtained after the larvae are dead.

The bacteria are confined exclusively to the gut of the host and do not penetrate the gut wall and invade the haemocoel. Microscopic and cultural examination of infected larvae showed that the blood remained sterile. Even in late stages of the disease, when gross damage to the epithelial cells of the midgut was evident, histological sections demonstrated that the bacteria were confined to the lumen of the gut and had not invaded the haemocoel. Usually, even when the host larvae were moribund or dead, secondary bacteria did not invade the haemocoel, but rarely they did and then caused a typical septicaemia.

The bacterial infection caused changes in the pH of the gut of the host. These were followed by pH measurements with the one-drop glass electrode and by a variety of colored pH indicators. In the healthy larvae of instars three and four, the pH of the regurgitation fluid and of the foregut varied from 6.5 to 6.8. In the early stages of infection, when only small numbers of vegetative rods were present, the regurgitation fluid had a pH of 7.7 to 7.8; this increased to 9.0 or 9.2 at the height of infection and dropped again to 7.7 or 8.0 as the insect shortened and the foregut was emptied. In the healthy midgut, the pH varied from 9.2 to 9.8, being highest about the center of the gut; in infected insects the pH was lower than normal and dropped to 8.6 or 8.8 at the height of infection. The pH of the hindgut in healthy larvae of instars three and four varied from 6.5 to 6.7 and did not change appreciably during infection. The pH of the blood of healthy larvae was from 6.7 to 6.8 and also did not change during infection.

Experimental Infection

M. pluviale

Preliminary experiments (Table I) showed that the first four instars of *M. pluviale* became infected by feeding on the bacterial spores and that doses as low as 950 spores per larva initiated infection in a lot of 25 larvae reared together. None of the larvae in an infected lot pupated. Some died from causes other than infection by *Bacillus* sp.; these included abnormal molting,

bacterial septicaemia, and a polyhedral virus disease. High mortality from other causes was a result of naturally occurring virus infection. The larva used in these tests were collected in the field early in the season in the first or second instar and showed no sign of natural infection with *Bacillus* sp.; thus the control larvae were free from infection with *Bacillus* sp. and most of them pupated.

TABLE I

AVERAGE PERCENTAGE MORTALITIES OF *Malacosoma pluviale* LARVAE, REARED IN LOTS OF 25, AFTER INGESTION OF VARIOUS DOSES OF SPORES OF *Bacillus* SP.

Dose, spores per larva	Number of replicate lots	Mortality, *Bacillus* sp.	Mortality, other causes	% pupated
First instar				
95,000	7	90	10	0
0	2	0	46	54
Second instar				
950,000	6	91	9	0
95,000	3	97	3	0
9,500	2	78	22	0
950	2	96	4	0
0	2	0	2	98
Third instar				
950,000	1	96	4	0
95,000	2	94	6	0
9,500	1	96	4	0
950	1	100	0	0
0	1	0	12	88
Fourth instar				
95,000	2	100	0	0
0	1	0	8	92

As infected larvae void bacterial rods and spores within 24 hours after infection, the larvae of an experimental lot are continuously exposed to cross-infection if any members become diseased, and the dose obtained through cross-infection may be much greater than the original dose. Therefore groups of larvae exposed to a graded series of doses do not respond with a graded series of mortalities and the LD_{50} cannot be determined.

Further experiments on dosage were made on fourth-instar larvae reared individually in vials in samples of 10 (Table II). Doses as low as 140 spores per larva produced infection and mortality of 100% in some samples and thus the experimental doses were too high to allow the LD_{50} to be determined. However, the occurrence of some disease in the controls reduces the reliance that can be placed on this figure. The experimental larvae were collected in the field later in the season than those for the preliminary experiments, and apparently some were infected with *Bacillus* sp. and transmitted the infection to other members of the web during shipment before individuals were confined to separate vials. After ingestion of low doses of 140 spores, larvae became moribund in a mean time of 6.0 days; after doses of 140,000 spores, they became moribund in 5.3 days. Thus the higher doses produced only a minor increase in the speed at which the disease progressed.

TABLE II

AVERAGE PERCENTAGE MORTALITIES OF FOURTH-INSTAR *Malacosoma pluviale* LARVAE, REARED INDIVIDUALLY IN SAMPLES OF 10, AFTER INGESTION OF VARIOUS DOSES OF SPORES OF *Bacillus* SP.

Dose, spores per larva	Number of replicate samples	Mortality, *Bacillus* sp.	Mortality, other causes	% pupated
140,000	3	97	3	0
14,000	3	97	3	0
1,400	3	97	3	0
140	3	97	3	0
0	2	35	15	50

Fifth-instar larvae were also susceptible to infection. An apparently healthy population from a single web was divided into two groups, one serving as a control the other being fed a dose of 140,000 spores per larva. Mortality in the treated group was 98% but the control group also developed the disease either from contamination in the laboratory or from the presence of a few infected larvae that were overlooked on first examination; 70% died with the disease and the remainder formed abnormally small cocoons most of which did not produce adults.

Isolations were made of a number of other bacteria during the attempts to culture *Bacillus* sp. from suspensions of spores. As it was possible that the morphology of *Bacillus* sp. would be much different in artificial culture than in the host, some of these could not be dismissed without further tests, although the number of colonies that appeared on plates seeded with spores was several thousand times less than expected if the colonies had resulted from growth of the spores. Six bacterial species occurred in cultures with considerable frequency and bore some resemblance to *Bacillus* sp. Type 1-A was a large, Gram-variable, slowly motile rod of dimensions 4 × 1 μ. Type 1-B was most similar to *Bacillus* sp.; it was a large, somewhat twisted, motile rod of dimensions 6–10 × 1 μ and belonged to the genus *Bacillus*; it stained Gram-positively only when very young, and bore spores centrally without bulging. Type 1-C was a motile, Gram-variable rod of dimensions 3–10 × 1 μ and showed considerable twisting and distortion of the rods. Type 2-C was a typical culture of *Bacillus cereus* F. & F. Type 3-A was a slowly motile, Gram-negative rod of dimensions 6–10 × 1 μ, bearing terminal spores with distinct bulging of the sporangia; it was very similar morphologically to *Bacillus sphaericus* Neide. Type 3-B was a motile, Gram-positive rod, oval in shape, with dimensions 2–2.5 × 1 μ. Each of these six bacteria was fed to a different population of 25 second-instar *M. pluviale* larvae at the following doses: 1-A, 320,000 bacteria per larva; 1-B, 320,000 and 32,000 bacteria per larva; 1-C, 140,000 bacteria per larva; 2-C, 730,000 bacteria per larva; 3-A, 700,000 bacteria per larva; 3-B, 180,000 bacteria per larva. All seven treated populations remained healthy for a period of 2 weeks and molted to the fourth instar. Thus none of these bacteria was identical with *Bacillus* sp.

M. americanum

Infection experiments with *M. americanum* were conducted on lots of 25 larvae, and are therefore subject to the same faults as those performed with *M. pluviale*, i.e., the doses were too high and cross-infection within the lot resulted in nearly 100% mortality (Table III). Some larvae had been attacked by braconids before collection and these are not included in the figures. Mortality from other causes included poor molting, bacterial septicaemia, and, chiefly, virus disease.

TABLE III

AVERAGE PERCENTAGE MORTALITIES OF *Malacosoma americanum* LARVAE, REARED IN LOTS OF 25, AFTER INGESTION OF VARIOUS DOSES OF SPORES OF *Bacillus* SP.

Dose, spores per larva	Number of replicate lots	Mortality, *Bacillus* sp.	Mortality, other causes	% pupated
Second instar				
5,200,000	1	92	8	0
3,000,000	2	72	28	0
520,000	1	100	0	0
0	2	0	18	82
Third instar				
5,200,000	2	70	24	6
3,000,000	1	96	4	0
520,000	2	94	6	0
0	2	0	28	72

M. americanum was susceptible to infection, at least at high doses and in the early instars. The course of the infection in *M. americanum* is very similar to that in *M. pluviale*. The bacteria multiply in the gut, chiefly in the midgut, and sporulate there. The larvae display the same symptoms of infection, including the characteristic copious regurgitation, diarrhea, and progressive shortening. The larvae empty the gut before becoming moribund but the faecal discharge lacks the intense, rusty-brown color characteristic of *M. pluviale*. The difference in color may be due to a difference in food. Larvae of *M. americanum*, in later stages of infection, have one symptom not shown by *M. pluviale*: in larvae with intense diarrhea that are emptying the gut, the rectum is frequently everted through the anus, and the white internal wall of the rectum shows in sharp contrast to the colored exoskeleton of the host.

M. disstria

Infection experiments on *M. disstria* were conducted on lots of 25 larvae in a manner similar to those on *M. pluviale* (Table IV). *M. disstria* suffered from virus disease, from the attack of tachinid parasites, and, more rarely, from bacterial septicaemia and the inability to molt.

M. disstria was highly resistant to infection. Some larvae apparently escaped infection and the bacteria could not be demonstrated in the gut. In other larvae, the bacteria multiplied and sporulated and the infected insects

TABLE IV

AVERAGE PERCENTAGE MORTALITIES OF FOURTH-INSTAR *Malacosoma disstria* LARVAE, REARED IN LOTS OF 25, AFTER INGESTION OF VARIOUS DOSES OF SPORES OF *Bacillus* SP.

Dose, spores per larva	Number of replicate lots	Mortality, *Bacillus* sp.	Mortality, other causes	% pupated
1,000,000	1	28	0	72
500,000	1	8	42	50
400,000	3	0	16	84
0	2	0	10	90

voided wet faeces containing numerous spores. In infected insects, the frass pellets characteristically formed long chains, the individual pellets being connected partly by the peritrophic membrane and partly by the chitinous intima of the hindgut. In healthy insects, the membranous covering of the frass pellets breaks between each pellet, which is deposited singly. After becoming infected, some larvae succumbed to the disease and died in the typical short condition shown by the other species, but others recovered from the infection, spun cocoons of normal size, pupated normally, and produced normal adults.

Discussion

In view of the fact that the bacterium has not been cultivated in pure culture on artificial media and thus Koch's second postulate has not been fulfilled, some of the other evidence that *Bacillus* sp. is indeed the causal agent of the disease has been presented in more than normal detail and may be summarized as follows:

(*a*) *Bacillus* sp. has been demonstrated microscopically in large numbers in all larvae that showed symptoms of disease. It has not been found in healthy larvae whose fore- and mid-guts are remarkably free of bacteria of any kind. The *Bacillus* exists in a pure or very nearly pure state in the fore- and mid-guts of diseased larvae; the microscopic appearance of the bacterium is distinctive and the range of sizes and forms shown by individual bacteria is consistent with the view that the individuals form a single species rather than a mixture of two or more species. The gut contents of diseased larvae containing huge visible numbers of bacteria did not produce or produced only rarely any growth on culture plates—further indication that the bacterium existed in pure culture in the host. The six bacteria that were isolated rarely from diseased insects and that bore some resemblance to *Bacillus* sp. did not produce disease when fed in massive doses. Thus Koch's first postulate has been fulfilled.

(*b*) The disease has been experimentally produced by feeding suspensions of the spores to susceptible larvae. Though raw suspensions contained a small number of viable contaminating organisms most of these were eliminated by heat and chemical treatments. Spore suspensions were infectious after being diluted up to 10,000 times; the probability that an unidentified or

unseen infectious agent was mixed with the spores in doses of 0.01 ml. at this dilution is extremely remote and no viable organisms could be demonstrated by culturing. Thus Koch's third postulate has been fulfilled.

(*c*) The bacterium occurs in huge numbers in the guts of larvae experimentally fed small doses of spores. It can be identified microscopically but not culturally and thus Koch's fourth postulate has been met in part.

The weight of evidence indicates that *Bacillus* sp. is the causal agent and that the disease is not caused by some undetermined factor.

The disease has a number of unusual features. The causal bacterium is unusual in that the vegetative rod grows in diameter as well as in length during formation of the spore and thus accommodates the spore without bulging. The bacteria grow and multiply chiefly in the midgut and to a lesser extent in the foregut of the host, but apparently do not multiply in the hindgut and never invade the haemocoel at any stage of the infection. This is in contrast to most bacterial infections of insects, in which the bacteria multiply principally in the haemocoel, and explains in part the dry, mummified condition and lack of putrefaction of larvae killed by the disease. The mummified appearance of dead larvae is also a result of extreme water loss during infection. Infected larvae lose water through excessive regurgitation and diarrhea and do not replace it by feeding. Water lost from the gut is replaced by fluids from the blood and tissues, as indicated by progressive reduction of blood volume and pressure. The drop in the pH of the contents of the midgut may be due to the passage of body fluids at a lower pH across the midgut membrane. The drop in pH may also be due to excessive mixing of the contents of the fore- and mid-gut through failure of the cardiac valve. The passage of fluids from the midgut to the foregut is indicated by the rapid rise of the pH of the foregut contents during the infection.

Growth and multiplication of the bacteria in the gut of the host seriously affect its ability to maintain a normal water balance and the direct cause of death is dehydration. In this respect the disease is somewhat analogous to bacillary dysentery in man. Death cannot be delayed by rearing the insects in an atmosphere of 100% humidity, indicating that desiccation through evaporation is a negligible factor. Most of the water is lost by diarrhea. In the tent caterpillar, as in other Lepidoptera, the blind ends of the Malpighian tubules coil about the wall of the rectum and with two rectal membranes form an organ whose apparent function is the resorption of water from the faeces. In infected insects this function is impaired.

The most striking symptom of the disease is the shortening of infected larvae. The shortening may be partly a result of desiccation and consequent reduction of volume, but is mainly due to muscular contraction, chiefly of the longitudinal muscles. Contraction of the intersegmental muscles telescopes the abdominal segments and shortens the larvae. At the same time the longitudinal muscles of the gut, particularly those of the midgut, contract so that the length of the midgut is reduced to one-quarter or less of the normal, and the wall is thrown into many deep folds.

As the infected larva regurgitates excessively and voids wet faeces, it is an efficient mechanism for the distribution of the disease throughout the members of a web. Extreme contraction of the gut muscles, shortly before the larva becomes moribund, empties the gut of most of the bacteria, and the larva excretes a fluid rich in spores. Thus moribund and dead larvae are poor sources of spores. This is in contrast to bacterial diseases of the haemocoel in which the bacteria are trapped until the host decomposes and the living infected larva is not a good source of infection for its fellows.

Though this is the first report of a disease in insects caused by a sporeforming bacterium that is confined exclusively to the gut, similar diseases may have been observed and attributed to starvation rather than to infection. As the symptoms are similar to those commonly associated with starvation and as the causal bacteria are not culturable on ordinary media and exist in small numbers in dead hosts, examination of the insects after death may fail to reveal the connection between the bacteria and the disease. For example, Bucher (2) classified as "starved" some larvae of *Choristoneura murinana* (Hbn.) that became very short before death; these larvae may have been infected with a bacterial disease similar to that of the tent caterpillars.

As about a week elapses between infection and death, the period of the disease may overlap the period of a larval instar. Larvae infected towards the end of an instar may molt and die in the following instar, but if they are infected towards the beginning of the instar they die before molting though the instar may be prolonged. Mature larvae infected shortly before spinning may form abnormally small cocoons; most of these die before pupating, some die as pupae, and only a few reach the adult stage. In larvae that die after spinning, few, if any, of the bacteria can be found in the gut, so that multiplication of the bacteria ceases when the host forms a cocoon. Yet many of these larvae die from the effects of previous development of the bacterium.

The fact that growth of the bacteria in the gut disturbs the water balance and causes extreme muscular contraction indicates that the bacteria produce a toxin. The production of a toxin is also suggested by the death of some larvae after they spin a cocoon and by the relative resistance of *M. disstria* to the disease, even in individuals that support multiplication of the bacteria in the gut. The fact that the symptoms become evident about the time that some of the bacteria are sporulating suggests that an endotoxin is liberated during autolysis of the mature sporangia. Clear demonstration of toxin production will depend on the successful culture of the bacteria. Histological studies of infected larvae are in progress to determine if changes in specific tissues can be correlated with the progress of the disease.

Observations by Mr. J. H. McLeod (in litt.), now of the Belleville laboratory, indicated that the disease was present in field populations of *M. pluviale* in the Fraser Valley, B.C., in 1954 and 1955. He reported that field-collected lots reared in the laboratory suffered a high mortality from the disease. No survey has been made to determine if *M. americanum* in Ontario is naturally infected by a similar or identical disease.

Acknowledgments

The author gratefully acknowledges the technical assistance of Miss C. M. Rutherford. Larvae of *M. pluviale* were collected through the courtesy of Mr. J. H. McLeod.

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CORRELATION BETWEEN BACTERIAL NUMBERS AND ORGANIC MATTER IN A FIELD SOIL[1]

P. H. H. Gray and R. H. Wallace

Abstract

The numbers of bacteria, with actinomycetes, were found to be significantly correlated with organic matter in field plots treated annually with straw, and straw with phosphate, followed by the ploughed-in crop of each plot. The treatment with straw significantly increased the numbers of microorganisms as well as the organic matter content in the plots during 3 years.

Evidence has recently been presented that bacterial numbers, estimated by the plate method, may be correlated with carbon dioxide evolved from a field soil (3). This paper presents evidence that bacterial "plate" numbers may also be correlated with organic matter in the same field soil. Examples of such correlations are scarce. Gray and McMaster (1) and Gray and Taylor (2) found that bacterial numbers were related to organic carbon, and with loss on ignition, in separate horizons of virgin podsol soils of Quebec and that bacterial activities such as hydrolysis of urea and oxidation of soil nitrogen were also related to the amount of organic matter. Jensen has shown (4) that bacteria and "organic matter" (loss on ignition) were significantly correlated in 50 soils in New South Wales. He has also shown (5) that bacterial cells estimated by a direct method were correlated with "organic matter" in another 13 soils of three different kinds. He stated that there was a "general, although by no means proportional increase with increasing content of organic matter". Analysis of his figures shows that the correlation coefficient r was 0.9196, which, for $n = 11$, is highly significant. Bacterial numbers by the plate method with dextrose–casein agar were not so closely related, which might be expected in such varied soils. It should be noted, though, that "organic matter" in Jensen's work was actually "loss on ignition", which may or may not be equivalent, depending upon the similarity or otherwise of the soils. It is preferable to attempt to assess the relationship of a biotic factor in soil with the food supply in soils of the same physical structure, or in one soil in which the food supply shall be the only or main variable. Many experiments have been made in which food material such as sugar, cellulose, straw, or other plant residues have been added to a soil; changes in the biotic balance have then been assessed by determining the evolution of carbon dioxide or by estimating the numbers of microorganisms during the period of most rapid decomposition. Such experiments have usually been done in a laboratory or in a glasshouse, equivalent to horticultural hothouse conditions. The results reported in this paper were derived from a field

[1]Manuscript received April 18, 1957.
Contribution from the Faculty of Agriculture, McGill University, Macdonald College, Macdonald College P.O., Que., Canada. Macdonald College Journal Series No. 410.

experiment; a description of the field conditions has been given in the previous paper (3). An analysis of the soil, a Chicot fine sandy loam, has been provided by Lajoie and Baril (6).

On September 8, 1956, samples were taken from each of the 20 field plots, which had received the treatments in the spring. The numbers of bacteria, with actinomycetes, were estimated from colonies in plates of soil extract agar, incubated at 22° for 5 days; the results are shown in Table I.

TABLE I

NUMBERS OF BACTERIA, WITH ACTINOMYCETES, MILLIONS PER G., AND ORGANIC MATTER, %, IN SAMPLES OF SOIL FROM THE EXPERIMENTAL PLOTS, SEPTEMBER 8, 1956

Plot and replicate	Treatment	Bacteria	Organic matter
A 1	Fallow	12.46	6.07
2		8.66	5.89
3		9.44	6.14
4		11.10	5.80
B 1	Crop only	14.43	6.28
2		11.90	6.21
3		10.40	6.28
4		14.59	5.96
C 1	Straw; crop	18.19	7.20
2		15.11	6.90
3		18.24	6.86
4		11.36	6.76
D 1	Phosphate; crop	19.22	6.35
2		13.86	6.28
3		12.50	6.17
4		13.63	5.48
E 1	Straw and phosphate; crop	15.44	6.76
2		18.35	6.83
3		13.61	6.51
4		16.49	6.38
Average		*13.95*	*6.35*

The chi-square index of dispersion of colonies showed that the method yielded reliable mean numbers from each sample. There were also no significant differences between replicate samples from any one treatment. The organic matter was determined in duplicate samples by the Walkley-Black method; the replicates of these results were found to be in close agreement, and the means are given in Table I. The moisture, which was uniform over the whole area, was determined by means of an infrared soil moisture balance. Correlation between bacterial numbers and the organic matter was high, r being + 0.5689 for $n = 18$; $P < .01$. The correlation is shown in the diagram in Fig. 1. A similarly high correlation was found when the analysis was made with the average values from the four plots of each treatment. It may be concluded that the differences in bacterial numbers were significantly similar to those of the organic matter.

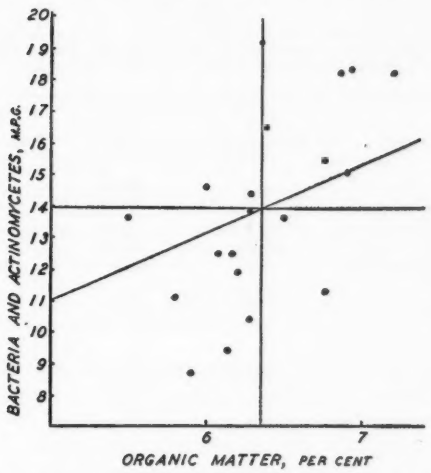

FIG. 1. Scatter diagram showing correlation between numbers of microorganisms (plate count) and organic matter in 20 field plots.

Discussion

It was stated in the previous paper (3) that the distribution of bacteria was uniform over the whole area before the plots were first treated in 1953. There was also no significant difference in the organic matter content in samples of soil from the plots. The treatments with straw, and straw with phosphate, stimulated the growth of bacteria and the evolution of carbon dioxide even during 1953 and were effective also in 1954 and 1955. The crops were ploughed into the soil in the early fall of each year, so that any differences among bacteria of the cropped plots in 1953 could be ascribed to the extra food provided by straw becoming available during the summer.

Differences now found in the organic matter content may be ascribed to the accumulation of undecomposed residues from the straw applied each spring. It is also possible that the treatment with straw has increased the activity of the nodule bacteria, giving rise to increased residues from the crops, but there are as yet no data to demonstrate that. The parallel increase in bacterial numbers was most likely caused by the annual addition of fresh decomposable material. The decomposable material being stored in the microbial cells may fluctuate from year to year, so that we might expect to find a significantly increased microbial population only after the treatments become reflected in increased crops and their residues. The results so far show that the bacterial numbers in the plots receiving straw are already significantly higher than in the other cropped plots. The annual levels of bacterial numbers in the separate plots during 1953–1955 are shown in Table II.

The average of the numbers in the straw treated plots is 23 millions per gram and that in the other two cropped plots 17; the difference is significant at the 98–99% level. The straw treatment has also significantly increased

TABLE II

NUMBERS OF BACTERIA, WITH ACTINOMYCETES, MILLIONS PER G.; AVERAGES FROM EACH TREATMENT IN 1953, 1954, AND 1955

Plot and treatment	1953 (3 samplings)	1954 (7 samplings)	1955 (3 samplings)	Average
A, fallow	16.3	19.8	19.8	*18.3*
B, crop only	14.9	19.1	16.3	*16.8*
C, crop; straw	20.8	21.6	27.7	*23.4*
D, crop; phosphate	15.4	23.5	12.6	*17.2*
E, crop; straw and phosphate	18.9	25.6	25.1	*23.2*
Average	*17.5*	*21.7*	*20.3*	

the organic matter content; this might be inferred from the correlation of the latter with the bacterial numbers; it has been confirmed by an analysis of variance and the t test for significance.

Acknowledgment

The authors are grateful to Dr. W. A. DeLong for analysis of organic matter and to Mr. R. A. Dawkins for technical assistance. This and the previous paper (3) constitute part of the work done in collaboration with the Department of Agronomy, Macdonald College, under grants from the Quebec Department of Agriculture.

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THE REMOVAL OF NON-SPECIFIC COMPONENTS FROM THE SOLUBLE ANTIGENS OF INFLUENZA AND MUMPS VIRUSES[1]

JOHN R. POLLEY

Abstract

An investigation has been made of methods to remove the non-specific complement-fixing components which sometimes occur in the routine preparation of virus soluble antigens. It was found possible to remove such components from influenza soluble antigens by (1) centrifugation for 1 hour at 36,000 *g* or (2) treatment of the antigen with 10% Norit, followed by uf. filtration. With mumps soluble antigen, this purification was accomplished by (1) centrifugation as above or (2) heating the antigen for 30 minutes at 70° C. at pH 9, followed by centrifugation at 9000 *g*.

Introduction

Soluble antigens of several viruses can be extracted from the virus-infected tissues (2, 6) and can be partially purified by relatively simple procedures (1, 3). For the laboratory diagnosis of a number of viral diseases, such as influenza, mumps, herpes simplex, soluble antigens are prepared in this Laboratory from the chorioallantoic membranes of specifically infected chick embryos. Occasionally these soluble antigens react non-specifically in the complement-fixation test. Antigen prepared similarly from the membranes of normal (uninoculated) chick embryos may also show non-specific complement-fixation. Furthermore, this non-specific reaction cannot be correlated with bacterial contamination of these antigens. In an attempt to eliminate non-specific reactions and to restore the specificity of the antigen, samples of these antigens have been subjected to various chemical and physical procedures.

Materials and Methods

For the preparation of the soluble diagnostic antigens, the chorioallantoic membranes from specifically infected chick embryos were harvested and centrifuged at 2000 r.p.m. for 15 minutes. The supernatant fluid was decanted and the packed membranes were washed by shaking with one volume of saline, then centrifuging again. The fluid was decanted and the membranes were macerated with four volumes of physiological saline in a Waring blendor for 3 minutes. The mixture was centrifuged again, the supernatant decanted and placed at 4° C. for 3–4 days. The precipitate which appeared on standing was removed by centrifuging at 5000 r.p.m. and the clear supernatant fluid was tested for the presence of soluble antigen. If it proved to be serologically specific and of satisfactory titer, it was treated to render it non-infective and lyophilized for stable storage (4). Antigens which were found to react non-specifically in the complement-fixation tests were subjected to various procedures in an attempt to render them specific.

[1]Manuscript received April 15, 1957.
Contribution from the Virus Laboratories, Laboratory of Hygiene, Department of National Health and Welfare, Ottawa, Canada.

TABLE I

EFFECT OF VARIOUS TREATMENTS OF AN INFLUENZA A (PR8) VIRUS SOLUBLE ANTIGEN ON NON-SPECIFIC COMPLEMENT-FIXATION REACTIONS

Treatment	CF titer with PR8 antiserum	CF titer with mumps antiserum
None (antigen control)	64	32
Dialyzed for 24 hours	64	32
uf. filtration	32	<2
10% Norit, then uf. filtration	32	2
10% Norit	32	16
Heated at 70° C. for 30 minutes at pH 9	16	4
Heated as above, then centr. at 5000 r.p.m.*	16	<2
Heated as above, then centr. at 10,000 r.p.m.	2	<2
Centrifuged at 5000 r.p.m. (2200 *g*)	64	8
Centrifuged at 10,000 r.p.m. (9000 *g*)	64	4
Centrifuged at 20,000 r.p.m. (36,000 *g*)	32	<2
Centrifuged at 30,000 r.p.m. (80,000 *g*)	4	<2

*All periods of centrifugation were 1 hour.

TABLE II

EFFECT OF VARIOUS TREATMENTS OF A MUMPS VIRUS SOLUBLE ANTIGEN ON NON-SPECIFIC COMPLEMENT-FIXATION REACTIONS

Treatment	CF titer with mumps antiserum	CF titer with PR8 antiserum
None (antigen control)	64	32
Dialyzed for 24 hours	64	32
uf. filtration	16	2
10% Norit, then uf. filtration	<2	<2
10% Norit	32	16
Heated at 70° C. for 30 minutes at pH 9	64	16
Heated as above, then centr. at 5000 r.p.m.*	32	<2
Centrifuged at 5000 r.p.m. (2200 *g*)	64	32
Centrifuged at 10,000 r.p.m. (9000 *g*)	64	16
Centrifuged at 20,000 r.p.m. (36,000 *g*)	32	<2
Centrifuged at 30,000 r.p.m. (80,000 *g*)	2	<2

*All periods of centrifugation were 1 hour.

Dialysis

Dialysis of non-specific antigens in the cold at various pH levels did not alter their behavior in the complement-fixation test, as is shown in Tables I and II. Nitrogen determinations (5) on the dialyzed samples showed that the amount of nitrogen removed by dialysis (about 15%) was not influenced by the pH at which the dialysis was conducted.

Filtration

Samples of non-specific soluble antigens of influenza and mumps viruses were passed through various filter papers, Seitz filters, and fritted glass filters of different porosities. The filtrates were examined by the complement-fixation test to determine whether any of the non-specific reaction had been removed. From Tables I and II it can be seen that uf. filtration (ultrafine fritted glass filter, Corning Glass Works) removed most or all of the non-specific complement-fixing activity from influenza virus soluble antigens. Unfortunately, however, the filters soon became obstructed so that the yield of purified filtrate was small. As a possible aid to uf. filtration, portions of antigens were adjusted to various pH levels and then filtered. There was no significant improvement in the results. In another experiment, samples of soluble antigens were shaken for 1 hour at room temperature with 10% (w/w) of Norit, then centrifuged at 3000 r.p.m. for 15 minutes. Portions of the clear supernatant fluids were filtered as before. These clear fluids passed through the filter more readily, so that the rate of filtration and the yield of purified filtrate were increased. With mumps virus soluble antigen, most of the complement-fixing activity was lost by uf. filtration, especially when there was prior treatment with Norit. When the fritted glass disks were primed by the passage of about 10 ml. of 1% albumen solution, influenza A soluble antigen treated with Norit passed through readily, leaving behind the non-specific component. With influenza B soluble antigen, priming the disk appeared to facilitate the passage of the non-specific component, and purification was decreased. When a sample of mumps soluble antigen was centrifuged at 10,000 r.p.m. (9000 *g*) for 1 hour and the supernatant fluid was then passed through the uf. filter, the filtrate gave non-specific complement-fixation to almost the same degree as the unfiltered material.

Adsorption

Attempts were also made to remove the non-specific components by adsorption. Various adsorbents, such as Celite, kaolin, Norit, and alumina were used in concentrations of 1–10% by weight. Each was shaken with samples of an antigen containing the non-specific component for 1 hour at room temperature. The mixture was then centrifuged at 3000 r.p.m. for 15 minutes and the clear supernatant fluids tested in the complement-fixation test. None of the adsorbents under these conditions effected a significant degree of purification of the soluble antigen. It was noted, however, that the sample treated with Norit was almost clear, suggesting that it could now be more readily filtered, as was found above.

Centrifugation

Since the previous experiments showed that the non-specific component did not pass through a dialyzing membrane and was retained by a uf. filter to a greater extent than the specific antigen, purification was attempted by

centrifugation. Samples of soluble antigens showing non-specific complement-fixation were centrifuged at various speeds for 1 hour, using the No. 40 rotor in a Spinco model L centrifuge. The supernatant fluids were removed and tested. Some typical results are shown in Tables I and II. As the centrifugal force is increased, the non-specific component is precipitated more readily than the specific antigen until at 20,000 r.p.m. (36,000 *g*) there is an effective purification of the antigens. The non-specific component could be recovered quantitatively in the resuspended precipitate. When centrifuged at 30,000 r.p.m. (80,000 *g*) most or all antigen was removed from the supernatant fluid and could be recovered from the precipitate. Evidently, the non-specific reactant is of greater weight than the specific antigens of influenza and mumps viruses and can be separated from them by high speed centrifugation.

Heating

Heating non-specific soluble antigens at various pH levels at temperatures of 56° C. and 70° C. did not produce significant selective destruction of the non-specific component. The variations in heat stability with different antigen preparations precluded the use of heat alone as a useful purification procedure. As seen in Tables I and II, influenza soluble antigen was less stable under heat treatment than was mumps antigen. When heating was followed by centrifugation, however, it did effectively remove the non-specific reactants from mumps virus soluble antigen. Heating the mumps soluble antigen at 70° C. for 30 minutes at pH 9 allowed the non-specific component to be removed by centrifuging at 5–10,000 r.p.m. rather than the 20,000 r.p.m. which was required otherwise. If, after heating, the pH of the antigen was lowered below pH 9, then on centrifugation all complement-fixing activity was removed from the supernatant fluid.

Discussion

Since dialysis did not alter the behavior of the antigens in the complement-fixation test it appears likely that the non-specific reaction is due to components of large molecular weight. This suggestion is supported by the observation that the non-specific components are precipitated at lower centrifugal forces than are the specific antigens of influenza and mumps. Since the specific antigen of influenza passes through the uf. filter while much of the mumps specific antigen is retained, it would appear to suggest further that the particle size of the soluble antigen of mumps is larger than that of influenza. However, after centrifugation, both the mumps specific antigen and the non-specific components pass readily through the uf. filter. These observations may indicate that with soluble antigens, such as influenza, which are purified by uf. filtration, the impurities are retained by adsorption to the fritted disk, rather than because of their particle size.

Thus it seems desirable to include the removal of non-specific components in the process of preparation of the soluble diagnostic antigens described above.

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ESSENTIAL AMINO ACIDS IN MICROORGANISMS[1]

F. REUSSER,[2] J. F. T. SPENCER, AND H. R. SALLANS

Abstract

The cells of 19 species of bacteria, actinomyces, and yeasts were analyzed for protein and essential amino acids. A rapid quantitative method for amino acid determination using one-dimensional paper chromatography was developed. The cellular protein from all species contained relatively high concentrations of lysine, somewhat lower concentrations of tryptophan and threonine, and very low concentrations of methionine. All of the 10 essential amino acids were found in each species tested, although individual differences in the relative and absolute amounts were observed.

Introduction

Recently, a number of studies were reported on the amino acid composition of bacteria and molds. Camien *et al.* (1) found that the amino acids of some lactobacilli remained nearly constant in cells cultured in a wide variety of synthetic media, and Freeland and Gale (2) found similar results in other microorganisms. Stokes and Gunness (10) point out that the amino acid composition of a microorganism is qualitatively and quantitatively a stable characteristic of the cell under fixed growth conditions, but that the amino acid content varies slightly among different strains and under different environmental conditions. Rao and Wadhwani (7) found that the composition of the protein of *Mycobacterium tuberculosis* varied according to the age of the cultures and the nature of the nitrogen source.

Williams (13) has pointed out that the problem of supplying adequate protein in the diets of the peoples of underdeveloped and overpopulated areas is one of the problems urgently requiring an early solution. Microbial proteins can be used as sources of some of the essential amino acids frequently lacking in the diets of these peoples (9, 11, 12) so that variations in the amino acid composition of microbial proteins are important. In addition, microbial proteins are a valuable livestock feed supplement (12). Thus a study of the essential amino acids of a number of species of microorganisms was undertaken.

Materials and Methods

Cultural Methods

Cultures were obtained from Miss Mary T. Clement, Division of Applied Biology, National Research Council, Ottawa; Dr. C. E. Chaplin, Division of Bacteriology and Dairy Research, Science Service Laboratories, Ottawa; Dr. A. C. Blackwood of this laboratory. The organisms were grown at 25° C. on a shaker rotating at 270 r.p.m. with ½ in. eccentricity. Where larger quantities of cells were required they were produced in submerged culture in a

[1]Manuscript received April 3, 1957.
Contribution from the National Research Council of Canada, Prairie Regional Laboratory, Saskatoon, Saskatchewan.
Issued as N.R.C. No. 4397.
[2]Postdoctorate Fellow, 1955–1957.

TABLE I

MEDIA

(Concentrations in g./liter unless otherwise indicated)

	Glucose	KH_2PO_4	$MgSO_4 . 7H_2O$	$FeSO_4 . 7H_2O$	$MnSO_4 . H_2O$	$ZnSO_4 . 7H_2O$	NaCl	$CaCl_2$	$(NH_4)_2SO_4$	Other constituents, per liter
Pseudomonas aeruginosa	10	5	0.02	0.0014	0.0009	0.001	5	0.05	4	
Acetobacter suboxydans	—	5	0.5					0.05		Tryptone 5 g., liver extract 1 g., mannitol 10 g., $CaCO_3$ 1 g. (latter added after sterilization)
Aerobacter aerogenes	10	5	0.02	0.0014	0.0009	0.001	5	0.05	4	Thiamine 50 μg., *p*-aminobenzoic acid 250 μg.
Serratia marcescens	2.5	5							0.5	NH_4Cl 0.5 g., $CaCO_3$ 1 g. (added after sterilization)
Bacterium cadaveris	10	5	0.02	0.0014	0.0009	0.001	5	0.05	4	
Azotobacter spp.		0.4	0.2	0.015			0.2			Mannitol 15 g., $CaSO_4$ 0.1 g., K_2HPO_4 1.6 g., $NaMoO_4$ 0.001 g.
Streptomyces sp.	10	2	0.02	0.0014	0.0009	0.001	5	0.05		$(NH_4)_2HPO_4$ 4 g.
Yeasts	60	4	0.5						5	Corn steep liquor 2 g.
All others										Peptone (Difco) 10 g./liter

30 liter stirred fermentor. Where possible the organisms were grown on a chemically defined medium. The media used are shown in Table I. After 24 to 40 hours' growth the cells were harvested with a Sharples supercentrifuge, were washed with distilled water, and dried under vacuum at 60° C.

Hydrolysis of Protein

The cell protein was hydrolyzed by refluxing 2 g. of cells in 75 ml. of 6 *N* HCl for 5 hours in the presence of stannous chloride as described by Kofrányi (3). The stannous chloride prevents to a considerable extent the formation of "humin" in the hydrolyzate so that nearly colorless hydrolyzates are obtained. The hydrolyzates were evaporated to dryness and the hydrochloric acid removed by repeated addition of water and evaporation at reduced pressure. The residue was dissolved in a solution of 5% isopropanol in water and aliquots used for separation of the amino acids. Samples for chromatography on basic papers were adjusted with potassium hydroxide to pH 6–7.

Paper Chromatography of Amino Acids

This investigation required that a very large number of amino acid assays be carried out and after surveying possible methods a one-dimensional paper

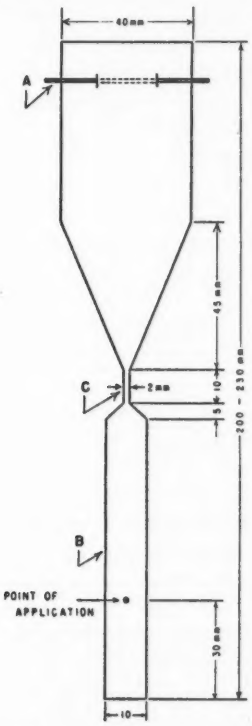

FIG. 1. Paper strip used for quantitative chromatography of amino acids.

chromatographic technique was used. The circular-ascending method described by Matthias (5) was adapted for quantitative determinations using buffered papers and solvents prepared by McFarren's (4) method. The amino acids were found in well-separated narrow bands when this method was used, and no difficulty with overlapping spots was encountered.

Filter Papers

Whatman No. 1 filter paper was cut in short strips (Fig. 1). The length of the basal section (B) was varied such that the amino acid with the lowest R_f value reached a position slightly past the bridge (C). The glass rod (A) rested on a rack in the chromatography tank, and could be used to support several paper strips.

Buffers

The buffers were approximately 0.067 *M* (4). The buffer of pH 2.0 contained 5 ml. of 0.067 *M* potassium chloride plus 10.66 ml. of 0.067 *M* hydrochloric acid, and the buffer of pH 8.4 contained 50 ml. of a mixture of boric acid and potassium chloride, 0.067 *M* with respect to each solute, and 8.55 ml. of 0.067 *M* sodium hydroxide.

Solvent Mixtures

Analytical grade solvents were used and all were redistilled except for *n*-butanol and acetic acid. The solvents used for the separation of the different amino acids were as follows:

Amino acids separated	Solvent	pH of buffer and paper
Lysine, threonine	Phenol – *n*-butanol – acetic acid – buffer (10: 10: 4: 20 by volume)	2.0
Histidine, valine, methionine, phenylalanine	*m*-Cresol (saturated with buffer)	8.4
Isoleucine, leucine, phenylalanine	Benzyl alcohol – tert. amyl alcohol – buffer (10: 10: 20)	8.4
Arginine	*n*-Butanol – acetic acid – H_2O (20: 3: 20)	—

The chromatograms were irrigated with solvent for 8–12 hours, removed from the chromatography jar, and dried. They were then replaced in the jar in the same position and again irrigated with solvent for 8–12 hours. This process was repeated two or three times, if necessary, to give wider separation of the bands of amino acids. After the final air-drying they were dipped in a solution of 0.5% ninhydrin in acetone containing 10% acetic acid and the color developed by heating for 10 minutes at 65° C. The colored bands were cut out and the color eluted by shaking them for 10–15 minutes in tubes containing 7 ml. of 75% ethanol. The color densities of the resulting solutions were determined with a Coleman spectrophotometer at a wavelength of 560 mμ and the amounts of amino acids calculated by reference to the appropriate standard curves. The accuracy of the method was 5–10%.

Determination of Tryptophan

Tryptophan is largely destroyed by acid hydrolysis severe enough to give complete breakdown of protein to amino acids and was therefore determined directly. The photometric method of Roth and Schuster (8) based on the xanthoproteic reaction was used. Optical densities of the solutions were measured at 425 mμ on a Coleman spectrophotometer and the tryptophan content of the unknowns calculated from a standard curve.

Determination of Total Nitrogen

Nitrogen was determined by the conventional micro-Kjeldahl method and protein calculated arbitrarily by multiplying by the factor 6.25.

Results and Discussion

The results are shown in Tables II and III. The protein content of the dry matter varied from 45.4% for the *Streptomyces* culture to 84.1% for *Alcaligenes viscosus*. The estimates of protein were based solely on total nitrogen content, and since the cells may contain some non-protein nitrogen, the values reported may be high.

The 10 essential amino acids studied were found in every hydrolyzate. The concentration of lysine varied from 4% to over 9% of the total protein. Lysine is especially important from the nutritional point of view and since the values reported show that these proteins were relatively rich in lysine, the microbial proteins could have a significant role in feed supplementation.

Tryptophan and threonine were found in somewhat lower concentrations than lysine. The amount of tryptophan was considerably higher than would be expected on the basis of previous reports (1, 6, 10). In the methods used by earlier workers part or all of the tryptophan may be destroyed by the hydrolytic procedures commonly used; however, since cystine, oxyproline, phenylalanine, glutathione, and some other compounds give a weak yellow color in the procedure used in these experiments, the results reported here may be slightly high. The originators of the method, however, claim that the error should not exceed 0.5%. Errors of this magnitude would not affect the generalization that the tryptophan content of these proteins of microbiological origin studied in this work was much higher than was found by other investigators.

All of the hydrolyzates were extremely low in methionine. The protein of *Bacterium cadaveris* had the highest methionine content with 2.8% of the total protein. Methionine was estimated in the protein hydrolyzates and since this amino acid is known to be partially labile under the conditions some losses may have occurred.

The other essential amino acids are less likely to be deficient in human and livestock nutrition and so are not discussed in detail.

All the organisms tested contained all 10 "essential" amino acids. They were all relatively rich in lysine, fairly rich in threonine and tryptophan, and low in methionine. In general, as was found by Mojonnier *et al.* (6) and by

TABLE II

ESSENTIAL AMINO ACIDS IN MICROORGANISMS

(The results are reported as mg. of amino acid per g. dry wt. of cells. All data represent averages of three determinations)

	Lysine	Tryptophan	Threonine	Methionine	Histidine	Valine	Isoleucine	Leucine	Phenylalanine	Arginine
Pseudomonas aeruginosa PRL F20	46	23	19	16	27	28	22	69	22	50
Acetobacter suboxydans PRL G1	43	18	17	12	23	24	25	56	25	35
Bacillus subtilis PRL B92	52	21	15	12	22	25	23	48	22	24
Bacillus polymyxa PRL B473	43	26	32	15	43	43	35	74	34	49
Aerobacter aerogenes PRL R4	29	27	8	6	4	16	10	26	++	19
Serratia marcescens PRL S2	47	26	19	13	23	28	15	52	33	37
Micrococcus pyogenes PRL M2	56	18	17	10	17	20	13	33	24	20
Micrococcus lysodeikticus PRL M140	61	26	20	15	38	25	17	32	20	41
Bacterium cadaveris PRL W1	52	22	17	22	37	36	23	63	33	49
Bacterium linens ATCC 9175	28	21	20	17	19	30	19	31	21	28
Rhizobium leguminosarum ATCC 10314	43	23	26	9	16	33	26	49	17	47
Corynebacterium fimi ATCC 8183	54	27	28	18	17	41	28	65	35	52
Alcaligenes viscosus ATCC 9036	53	20	35	14	17	32	33	81	42	49
Flavobacterium aquatile ATCC 11947	44	30	18	12	13	23	22	36	23	30
Azotobacter chroococcum DASS 319	43	25	25	11	43	39	26	52	28	61
Streptomyces sp.	25	16	11	+	11	17	14	20	+	21
Torulopsis utilis NRC 862	39	11	22	8	12	15	15	57	23	26
Candida arborea NRC 802	27	31	11	13	16	22	21	74	25	19
Torulopsis berlese NRC 858	44	19	23	10	27	26	25	89	27	38

+ Traces.
++ Contaminated with an unknown ninhydrin-positive compound.

TABLE III

ESSENTIAL AMINO ACIDS IN MICROORGANISMS

(The results are reported as per cent of crude protein (N × 6.25). All data represent averages of three determinations)

Organisms	Protein, % of dry weight	Lysine	Tryptophan	Threonine	Methionine	Histidine	Valine	Isoleucine	Leucine	Phenylalanine	Arginine
Pseudomonas aeruginosa PRL F20	79.3	5.8	2.9	2.4	2.0	3.4	3.5	2.8	8.7	2.8	6.3
Acetobacter suboxydans PRL G1	61.4	7.0	2.9	2.8	2.0	3.7	3.9	4.1	9.1	4.1	5.7
Bacillus subtilis PRL B92	67.1	7.7	3.1	2.2	1.8	3.3	3.7	3.4	7.2	3.3	3.6
Bacillus polymyxa PRL B473	76.3	5.6	3.4	4.2	2.0	5.6	5.6	4.6	9.7	4.5	6.4
Aerobacter aerogenes PRL R4	48.3	6.0	5.6	1.9	1.2	0.8	3.3	2.1	5.4	++	3.9
Serratia marcescens PRL S2	66.7	7.1	3.9	2.8	1.9	3.5	4.2	2.2	7.8	4.9	5.5
Micrococcus pyogenes PRL M2	57.6	9.7	3.1	3.0	1.7	3.0	3.5	2.3	5.7	4.2	3.5
Micrococcus lysodeikticus PRL M140	75.1	8.1	3.5	2.7	2.0	5.1	3.3	2.3	4.3	2.7	5.5
Bacterium cadaveris PRL W1	79.4	6.6	2.8	2.1	2.8	4.7	4.5	2.9	7.9	4.2	6.2
Bacterium linens ATCC 9175	68.7	4.1	3.1	2.9	2.5	2.8	4.4	2.8	4.5	3.1	4.1
Rhizobium leguminosarum ATCC 10314	72.8	5.9	3.2	3.6	1.2	2.2	4.5	3.6	6.7	2.3	6.5
Corynebacterium fimi ATCC 8183	83.1	6.5	3.2	3.4	2.2	2.0	4.9	3.4	7.8	4.2	6.3
Alcaligenes viscosus ATCC 9036	84.1	6.3	2.4	4.2	1.7	2.0	3.8	3.9	9.6	5.0	5.8
Flavobacterium aquatile ATCC 11947	66.3	6.6	4.4	2.7	1.8	2.0	3.5	3.3	5.4	3.5	4.5
Azotobacter chroococcum DASS 319	77.8	5.7	3.2	3.2	1.4	5.5	5.0	3.3	6.7	3.6	7.8
Streptomyces sp.	45.4	5.5	3.5	2.4	+	2.4	3.7	3.1	4.4	+	4.6
Torulopsis utilis NRC 862	50.0	7.8	2.2	4.4	1.7	3.2	3.0	3.0	11.4	4.6	5.2
Candida arborea NRC 802	51.2	5.3	6.1	2.1	2.5	4.5	4.3	4.1	14.5	4.9	3.7
Torulopsis berlese NRC 858	60.4	7.3	3.1	3.8	1.7	2.4	4.3	4.1	14.7	4.5	6.3

+ Traces.
++ Contaminated with an unknown ninhydrin-positive compound.

Stokes and Gunness (10), the amino acid compositions of the proteins of the organisms studied were qualitatively similar but there were some quantitative differences.

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THE CHEMOTHERAPEUTIC ACTIVITY OF A REACTION PRODUCT OF CYSTEINE AND IRON IN EXPERIMENTAL TUBERCULOSIS[1]

NORMAN A. HINTON AND J. KONOWALCHUK

Abstract

An unusual form of colloidal sulphur is precipitated when dilute solutions of cysteine hydrochloride and ferric ammonium citrate are mixed and autoclaved. The intravenous injection of this relatively insoluble particulate material is associated with a retardation in the progress of tuberculosis in guinea pigs and rabbits, and a change in the histology of the tubercle to a more proliferative and less destructive form. A considerable therapeutic effect is seen when infected rabbits are treated with a combination of streptomycin and this form of colloidal sulphur.

Introduction

The intracellular residence of *Mycobacterium tuberculosis* is a most important feature of the pathogenesis of tuberculosis. Many workers believe that it is within the monocytes or their derivatives, the epithelioid and giant cells, that the most critical battle between host and parasite is waged.

It would seem reasonable to assume that the most desirable form of chemotherapeutic agent which could be developed would be one which would enforce the normal lines of defence in the animal body and effectively prevent the prolonged residence of tubercle bacilli within the phagocytic cells of the host. The administration of such a drug would be associated not only with the attaining of effective levels in the extracellular fluids but also attaining these levels within the mononuclear cell.

This concept is not a new one and was stated clearly by Wells, DeWitt, and Long in 1923 (8), 15 years before any useful antitubercular agent had been discovered. "If we wish to destroy the tubercle bacilli in the body, we must consider, not only what chemicals can kill the bacilli in the test tube, but also which ones can penetrate the tubercle and pass through the wall of the phagocyte in which it is so often entrenched."

High intracellular concentrations of an antitubercular agent in the reticuloendothelial system in general and the bacilli-laden phagocytes in particular might be obtained by the intravenous injection of an agent having the physical characteristics of a colloidal dye or particular matter. This agent may be phagocytosed by reticuloendothelial cells, and provided that the activity of the drug was not impaired by intracytoplasmic physicochemical conditions, it could exert its antibacterial activity at the specific site of multiplication of the organism.

Workers at Oxford have recently reviewed this problem (6) and using the particulate antibiotic micrococcin have attempted to test the thesis that

[1]Manuscript received April 8, 1957.
Contribution from the Department of Bacteriology, Queen's University, and the Defence Research Board, Kingston Laboratory, Kingston, Ontario.

mononuclear cells can be specifically *fortified* against *M. tuberculosis*. The results of the treatment of experimental tuberculosis with suspensions of micrococcin (7) or with micrococcin–triton solution (1) were disappointing.

Konowalchuk *et al.* (3, 4) have shown that an unusual form of colloidal sulphur is precipitated when dilute solutions of cysteine hydrochloride and ferric ammonium citrate are mixed and autoclaved. This preparation forms a semistable suspension in water or saline consisting of rounded particles 1 to 2 μ in diameter which, unlike conventional colloidal sulphur, may be stored at 5° C. for many weeks without crystallization.

The substance exhibits considerable bacteriostatic activity *in vitro* against a variety of microorganisms and, in particular, inhibits the growth of *M. tuberculosis* in concentrations of less than 1 mcg./ml.

The purpose of this paper is to assess the *in vivo* antitubercular activity of this reaction product of cysteine and iron. Because it was originally thought that this form of colloidal sulphur was a complex of cysteine and iron, it will be referred to as complex for the purpose of distinguishing it from conventional colloidal sulphur.

The Effect of Complex on Established Tuberculosis in Guinea-pigs

The complex and colloidal sulphur were prepared and standardized as previously described (4). Eighty-seven guinea pigs ranging in weight from 300–500 g. were inoculated intraperitoneally with 1.0 ml. of a 1:1000 dilution of a culture of *M. tuberculosis* H37Rv grown for 10 days in Middlebrook and Dubos medium. Two weeks after the initiation of the infection the animals were divided into four groups:

(*a*) 21 untreated controls,
(*b*) 21 receiving 5 mg. of intramuscular streptomycin daily,
(*c*) 21 receiving 5 mg. of colloidal sulphur intracardially three times weekly,
(*d*) 24 receiving 5 mg. of complex intracardially three times weekly.

Therapy was discontinued after 3 weeks because of the risk entailed in intracardiac injection, and all animals were sacrificed and examined. The amount of tuberculous involvement of omentum, spleen, liver, and lungs was recorded as extensive, moderate, minimal, or none on gross examination.

TABLE I

THE EFFECT OF STREPTOMYCIN, COLLOIDAL SULPHUR, AND COMPLEX ON TUBERCULOSIS IN GUINEA PIGS

Type of therapy	Amount of tuberculous involvement of various organs																Number of animals
	Omentum				Spleen				Liver				Lung				
	+++	++	+	0	+++	++	+	0	+++	++	+	0	+++	++	+	0	
Control	16	1	2	2	19	1	0	1	10	4	0	7	5	0	0	16	21
Colloidal sulphur	15	1	2	3	16	4	1	0	9	2	1	9	10	0	0	11	21
Streptomycin	6	5	3	7	15	5	1	0	2	3	3	13	0	3	0	18	21
Complex	3	8	4	9	8	3	2	11	3	1	0	20	2	0	1	21	24

NOTE: +++ Extensive gross tuberculosis; ++ moderate; + minimal; 0 none.

A consideration of Table I indicates that the intravenous administration of complex has retarded the progress of tuberculosis in the guinea-pigs to a significant degree. However, colloidal sulphur, although a very close chemical relative of complex, can be seen to have allowed the disease to develop to a degree indistinguishable from that in the untreated group.

The Effect of Complex on Tuberculosis in the Rabbit

Thirty-five male rabbits of mixed breeds weighing about 3000 g. were challenged intravenously with 1.0 ml. of a 1:1000 dilution of a culture of *M. tuberculosis* var. *bovis* No. 30 grown in Middlebrook and Dubos medium for 10 days. The bovine strain used was a recent isolate obtained through the courtesy of Dr. Charles A. Mitchell (of the Animal Diseases Research Institute, Hull, Quebec) and was shown to be sensitive to the bacteriostatic activity of complex at limiting dilution at a level of 0.5 mcg./ml. Therapy was started the following day, the animals being divided into four groups:

(*a*) 10 untreated controls,
(*b*) 10 receiving 50 mg. of intramuscular streptomycin daily,
(*c*) 10 receiving 5 mg. of intravenous complex three times a week,
(*d*) 5 receiving 5 mg. of intravenous complex three times a week and 50 mg. of intramuscular streptomycin daily.

The disease in the untreated animals had progressed to a gross stage within a period of 4 weeks and at the end of this time all animals were sacrificed and post-mortem examinations performed. Sections were made of lung, spleen, liver, and kidney and stained with hematoxylin and eosin. The degree of infection was recorded numerically on the basis of gross and histological findings, from a maximum of 4, indicating extensive gross disease, to a minimum of 0, indicating no evidence of tuberculosis on microscopy.

The results as recorded in Tables II and III indicate that complex in this dosage does retard the progress of the disease to a limited extent. Microscopy, however, revealed that the complex had influenced the course of the disease more than is indicated by the numerical recording of the degree of infection.

The lesions in the lungs of control animals consisted of compact aggregations of epithelioid cells surrounded by moderate numbers of lymphocytes. Occasional giant cells were noted and early caseation necrosis was seen in a fair number of tubercles. Polymorphonuclear neutrophiles could be distinguished with ease about the periphery of the lesions.

The epithelioid cells in the pulmonary lesions of complex-treated animals were loosely packed, many demonstrating a stellate configuration. Less caseation was noted, giant cells were less frequent and polymorphonuclear cells were much less abundant than in comparable control lesions. The less destructive nature of the lesions in complex-treated animals was manifested in the ease with which the normal architecture of the lung could be made out even in grossly involved areas.

TABLE II

THE EFFECT OF STREPTOMYCIN, COMPLEX, AND A COMBINATION OF THE TWO ON TUBERCULOSIS IN THE RABBIT

Organ	Degree of tuberculosis: Untreated controls										Degree of tuberculosis: Complex treated										Degree of tuberculosis: Streptomycin treated										Degree of tuberculosis: Complex and streptomycin				
Spleen	4	4	4	4	4	4	4	4	4	3	4	4	4	4	4	3	4	4	3	3	2	2	2	2	2	2	1	1	1	1	1	1	0	0	0
Lung	4	4	4	4	4	4	4	4	3	3	4	4	4	3	3	4	3	3	3	3	2	2	2	2	1	1	1	1	1	1	1	0	0	0	0
Liver	3	3	3	3	3	2	2	2	2	3	3	3	2	2	2	2	2	1	1	1	2	2	1	1	2	1	2	1	1	1	0	0	0	0	0
Kidney	2	2	2	1	1	1	1	1	2	1	2	1	1	1	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Whole animal	13	13	13	12	12	11	11	11	11	10	13	12	11	10	10	10	9	9	8	7	6	6	5	5	5	4	4	3	3	3	2	1	0	0	0

NOTE: The degree of tuberculosis is graded numerically to possible maximum of 4 for each organ, and a total of 16 for each animal.

TABLE III

THE AVERAGE DEGREE OF TUBERCULOSIS IN RABBITS TREATED WITH STREPTOMYCIN, COMPLEX, AND A COMBINATION OF THE TWO

	Average degree of tuberculosis			
Organ	Control	Complex	Streptomycin	Complex and streptomycin
Spleen	3.9	3.6	1.6	0.4
Lung	3.8	3.4	1.4	0.2
Liver	2.6	1.9	1.4	0.0
Kidney	1.4	0.9	0.0	0.0
Whole animal	11.7	9.8	4.4	0.6
Index of infection	100%	84%	38%	5%

NOTE: The average degree of tuberculosis is recorded for each organ out of a possible maximum of 4, and for each animal out of 16.

Although all the organs of the streptomycin-treated animals revealed far less pathology than those in the complex-treated group, the lesions which were observed had an histologic structure indistinguishable from that seen in the control animals.

The results of therapy with streptomycin and complex combined are indeed striking. No gross disease was found in any of the organs and only after careful examination of a number of sections from each organ were small lesions found in the lungs and spleen of one animal and in the spleen of another.

Discussion

Previous work in this laboratory (2) indicated that complex possessed antibacterial activity which could be demonstrated not only *in vitro* but also in therapeutic trials in mice infected with pneumococci. The results recorded above indicate that this form of colloidal sulphur is a rather unique example of a relatively insoluble material which has measurable, although modest, antitubercular activity *in vivo*.

It is tempting to speculate that the striking results seen in the animals treated with both complex and streptomycin are a manifestion of the predominantly extracellular effect of streptomycin (5) together with the intracellular activity of the complex. More direct evidence would be necessary, however, if this thesis is to be seriously entertained.

The less destructive nature of the lesions seen in complex-treated animals may be due to a delay in the development of hypersensitivity, as was observed by Markham *et al.* in micrococcin-treated animals (7), and it may be that the streptomycin can more readily penetrate the loose proliferative structure of the tubercle in these animals with a concomitant increase in its effectiveness.

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STUDIES ON DIPICOLINIC ACID IN THE SPORES OF BACILLUS CEREUS VAR. TERMINALIS[1]

WILLIAM K. HARRELL AND EMIL MANTINI[2]

Abstract

Dipicolinic acid constitutes 12% of the dry weight of spores of *Bacillus cereus* var. *terminalis*.

Following a heat-shock treatment at 65° C. glucose is oxidized by the ungerminated spores, the amount of oxidation increasing with time of heating from 10 μl. per hour at zero time to 230 μl. per hour after heating for 60 minutes. Also during this treatment there is an increase in the amount of dipicolinic acid released from 2% of the total at zero time to 12.5% of the total after 60 minutes.

The possible relationship between this material and the enzymes of resting spores is discussed.

Introduction

The presence of dipicolinic acid (pyridine-2,6-dicarboxylic acid) in the spores of several species of aerobic bacilli has been well established. Powell (7), working with the spores of *Bacillus megaterium* and *Bacillus subtilis*, identified this compound and found it constituted approximately 15% of their dry weight. Perry and Foster (6) reported that dipicolinic acid made up approximately 5% of the dry weight of spores of *Bacillus cereus* var. *mycoides* and *B. subtilis*. This material is found only in the resting spore, being synthesized during sporulation and released on germination. As yet, it has not been reported in any other living matter, which suggests it may play a special and perhaps a specific role in these organisms. The fact that it is a major constituent of resting spores further emphasizes this possibility.

Recently Church (1) observed that following a heat treatment at 65° C. for 60 minutes, resting spores of *B. cereus* var. *terminalis* would metabolize glucose, gluconate, 2-ketogluconate, and pyruvate. Cell-free extracts of the spores oxidized only glucose and pyruvate at an active rate and these only when supplemented with diphosphopyridine nucleotide (DPN) and adenosine triphosphate (ATP). Although these enzymes are heat resistant in the intact spore, they become heat sensitive in the germinated spores and in extracts of the spores.

It is the purpose of this paper to examine a possible relationship between the release of dipicolinic acid and the initiation of enzymatic activities in ungerminated spores of *B. cereus* var. *terminalis*.

Materials and Methods

The spores of *B. cereus* var. *terminalis* used in most of this work were kindly supplied by Dr. H. O. Halvorson, Department of Bacteriology, University of

[1]Manuscript received February 27, 1957.
Contribution from the Department of Bacteriology and Public Hygiene, School of Medicine, West Virginia University, Morgantown, West Virginia, U.S.A.
[2]National Institutes of Health Medical Student Research Fellow.

Illinois. One batch consisted of spores maintained in the lyophylized state for approximately five years. A second batch had been maintained in a frozen state for approximately six months. The older spores contained approximately 5% germinated forms. These were washed three times with distilled water, before being used, to ensure the removal of any dipicolinic acid that might have been released during this germination. The spores stored for 6 months contained less than 0.5% germinated forms. Counts to determine the percentage germination were made before and after each experiment. It was not possible to detect an increase in the number of germinated spores following heat treatment or at the conclusion of the Warburg studies.

The production of fresh spore suspensions, the determination of viability, and the measurement of germination have been previously described by Church *et al.* (2). Unless otherwise indicated, 30 mg. of spores was used in the experiments. The suspensions were prepared by taking 10 ml. aliquots of a stock suspension containing 3 mg. of spores per ml.

In all cases, dipicolinic acid was measured as the calcium salt at 278 mμ using a Beckman DU spectrophotometer. The acid hydrolysis method of Perry and Foster (6) was employed for the determination of total or bound dipicolinic acid. For this work, the cells were boiled for 15 minutes in 3 *N* sulphuric acid, an aliquot of hydrolyzate extracted with 15 volumes of ethyl ether in a separatory funnel, and, after separation, the ether phase evaporated to dryness. The residue, containing the dipicolinic acid, was resuspended in a small volume of water and, after the addition of calcium chloride, measured in the Beckman. The amount of dipicolinic acid was then read from a standard curve prepared from graded amounts of pure dipicolinic acid subjected to the same procedure.

Standard Warburg methods were employed for the determination of glucose metabolism by ungerminated spores. In all experiments 0.5 mg. of glucose was employed as the substrate. Phosphate buffer (0.1 *M*), pH 7.2, was used to bring the total volume to 2 ml. in the reaction vessels. All reactions were measured at 30° C. with air as the gas phase.

The method of Church and Halvorson (2) was used to initiate enzymatic activity in the resting spores. This consisted of suspending the spores in phosphate buffer, pH 7.2, and heating at 65° C. for the desired length of time.

Results

Percentage of Dipicolinic Acid in Spores of B. cereus var. terminalis

The acid-hydrolysis method previously described was used for these determinations. Using 20 to 30 mg. dry weight of spores, it was found that dipicolinic acid constitutes 12% of the dry weight of these cells. It was not possible at any time to detect the presence of this material in the vegetative cells of these organisms. This is approximately the same value reported by Powell (7) for the spores of *B. megaterium*.

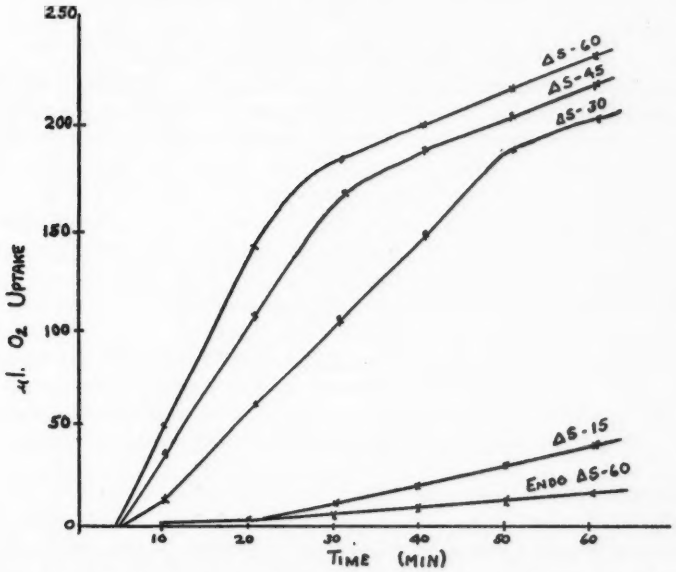

FIG. 1. The effect of heat on glucose oxidation by ungerminated spores of *B. cereus* var. *terminalis*. Spores suspended in phosphate buffer and heated for the indicated time (Δs). After centrifugation the spores were resuspended in a small volume of phosphate buffer, pH 7.2, and placed in the Warburg cups; 0.5 mg. of glucose was used as the substrate. The reaction was carried out at 30° C.

Relationship between the Release of Dipicolinic Acid and Glucose Metabolism in Ungerminated Spores

Spore suspensions were prepared in 0.1 *M* phosphate buffer and heated at 65° C. for 0, 15, 30, 45, and 60 minutes. At the end of each period of heating the suspension was rapidly cooled to room temperature and the spores separated by centrifugation at 4000 *g*. The supernatant was assayed for dipicolinic acid and the spores resuspended in a small exact volume of buffer for the Warburg studies. Fig. 1 shows the results of the enzymatic studies. It can be seen that there is an increase in both the rate and amount of oxygen taken up as the time of heating was increased from 0 to 60 minutes. Duplicate samples were run on the 0 and 60 minute cells. The total amount of dipicolinic acid in each stock solution was determined on one of the unheated tubes (zero time) while the second 60 minute sample was used to measure the endogenous respiration. These results are essentially the same as those reported by Church and Halvorson (3).

The amount of dipicolinic acid released during the various times of heating is shown in Table I. The supernatant from the unheated cells contained 2% of the total dipicolinic acid. This apparently represents loosely bound material which was released from the spores while they were standing at room temperature during the 60 minute test period. The spore suspensions heated

TABLE I

EFFECT OF HEAT ON GLUCOSE METABOLISM AND THE RELEASE OF DIPICOLINIC ACID IN UNGERMINATED SPORES

Time heated 65° C. (min.)	μl. O_2 per hr.	% of total DPA released
0	10	2.0
15	102	5.45
30	179	8.3
45	206	10.0
60	230	12.5

NOTE: Dipicolinic acid (DPA) measured as the calcium salt at 278 mμ in the Beckman spectrophotometer. Glucose (0.5 mg.) used as the substrate.

for 15, 30, 45, and 60 minutes released 5.45, 8.3, 10.0, and 12.5% respectively of the total dipicolinic acid contained in the spores. These data would indicate a possible parallel between the amount of dipicolinic acid released and an increase in the metabolism of glucose by the ungerminated spores.

Results with Freshly Harvested Spores

During the course of the investigation it was found that when freshly grown spores were prepared and treated in the same manner as the spores mentioned above, glucose oxidation could not be demonstrated. It was interesting to note that there was also no dipicolinic acid released from these spores after they were heated at 65° C. for 60 minutes. It has been recently shown by Murty and Halvorson (5) and Church and Halvorson (4) that there is a gradual change in the physiology of spores with age. This change could account for the metabolic activity in the intact aged spores and the absence of it in freshly grown spores treated in the same manner. These results also indicate a possible correlation between the release of dipicolinic acid and the initiation of enzymatic activity in ungerminated spores of this organism.

Discussion

As yet, it has not been possible to observe the metabolism of glucose in the spores of *B. cereus* var. *terminalis* without a release of dipicolinic acid. It has been known for some time that immediately following or, more likely, concurrent with germination, the enzymes of these cells become active. It was shown by Powell (7) and Perry and Foster (6) that dipicolinic acid was released from the spores during the germination process. From the work of Church (1) and Church and Halvorson (3), it is apparent that prolonged heat-treatment of spores of *B. cereus* var. *terminalis* changes these cells in some manner that enables them to metabolize glucose without germinating. The data reported here indicates a possible parallel between the release of dipicolinic acid and the "activation" in ungerminated spores of enzymes involved in the metabolism of glucose. As the time of heating at 65° C. is increased from 0 to 60 minutes, there is a concurrent increase in the rate and amount of glucose oxidation and the release of dipicolinic acid (Table I). It

is not known how heat "activation" affects the release of this material. Murty and Halvorson (5) reported that the enzymes involved in glucose metabolism in ungerminated spores of *B. cereus* var. *terminalis* were heat labile following a prolonged heat shock treatment. Thus, if dipicolinic acid is associated with the heat-resistance of enzymes in resting spores, it must be released only after the heated suspension is returned to a more favorable temperature. This could mean a change in the permeability of the spore coat to dipicolinic acid or a weakening, followed by a break of a chemical bond between dipicolinic acid and the enzymes present in the spores. From X-ray examination of resting spores of *B. megaterium*, Powell and Strange (8) could find no evidence of a crystalline structure of calcium dipicolinate and suggested that this material may be associated with heat-resistance through the formation of chelate linkages of calcium with protein. This latter possibility is now under present investigation.

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THE DEGRADATION OF 2-KETO-D-GLUCONATE-C^{14}, D-GLUCONATE-C^{14}, AND D-FRUCTOSE-C^{14} BY LEUCONOSTOC MESENTEROIDES[1]

E. R. Blakley and A. C. Blackwood

Abstract

2-Ketogluconate, gluconate, and fructose specifically labeled with C^{14} were fermented by *Leuconostoc mesenteroides*. Radioactive carbon dioxide was recovered from the fermentation of the C-1 labeled substrates, while methyl labeled lactic acid was recovered from the C-6 labeled compounds. The results indicate that the metabolism of these compounds and that previously reported for glucose are similar. This organism can be used to degrade these compounds for the determination of C^{14} distribution.

Introduction

A previous publication from this laboratory (4) demonstrated that the products from the metabolism of 2-keto-D-gluconate, D-gluconate, and D-fructose by *Leuconostoc mesenteroides*, PRL L33, were similar to the products obtained when glucose was metabolized by this organism (8). Since this culture has been used successfully in the degradation of labeled glucose (2, 3, 7), xylose (1, 2), and xylulose (14), the determination of the carbon atom relationships between the substrates 2-ketogluconate, gluconate, and fructose, and their metabolic products, was undertaken. The results suggest that these labeled substrates are degraded by a metabolic pathway similar to that for glucose and thus the specific degradation of radioactive 2-ketogluconate, gluconate, and fructose can be accomplished.

Materials and Methods

Fermentation Conditions

Leuconostoc mesenteroides, PRL L33, was adapted to the specific substrate and then grown as described previously (4). The fermentation of gluconate and 2-ketogluconate was satisfactory with the growing culture under conditions previously used (4), but was not satisfactory with resting cells under conditions used for glucose and xylose degradation (1, 2). Labeled fructose was fermented by either method.

Substrates

Calcium-D-gluconate-1-C^{14}, prepared by the cyanohydrin reaction, was provided by Dr. A. C. Neish of this laboratory. Potassium-D-gluconate-6-C^{14} was prepared by the oxidation of glucose-6-C^{14} according to the procedure of Moore and Link (12). Potassium-2-keto-D-gluconate-1-C^{14} and potassium-2-keto-D-gluconate-6-C^{14} were purchased from Merck and Co., Ltd., Montreal.

[1]Manuscript received April 12, 1957.
Contribution from the National Research Council of Canada, Prairie Regional Laboratory, Saskatoon, Saskatchewan.
Issued as N.R.C. No. 4393 and as Paper No. 245 on the Uses of Plant Products.

The potassium-2-ketogluconate-6-C^{14} was recrystallized with added carrier to remove a radioactive contaminant. Fructose-1,6-C^{14} was prepared by the oxidation of mannitol-1,6-C^{14} by *Acetobacter suboxydans*, PRL G3, and purified by Dr. A. S. Perlin of this laboratory (5).

Separation and Degradation of Products

The completed fermentations were acidified and fractions containing the carbon dioxide, ethanol, and organic acids were obtained (4). The acetic and lactic acids were separated on a silicic acid column (13). Ethanol was oxidized with sodium dichromate (13) to acetic acid, which was recovered by steam distillation and was combined with the acetic acid obtained from the silicic acid column. Since radioactivity was not expected in the acetic acid fractions, they were not degraded further. Similarly, when no radioactivity was expected in lactic acid, it was combusted directly. Otherwise lactic acid was converted to carbon dioxide and acetic acid (11) and the acetic acid degraded by the method of Phares (14).

All samples were converted to carbon dioxide and the radioactivities measured in a proportional counting apparatus similar to that described by Buchanan and Nakao (6).

Results and Discussion

The distribution of radioactivity among the products resulting from the fermentation of 2-ketogluconate, gluconate, and fructose by *Leuconostoc mesenteroides* is shown in Table I. Essentially all the radioactivity from the fermentation of 2-ketogluconate-1-C^{14} and gluconate-1-C^{14} was recovered in the carbon dioxide fraction whereas from 2-ketogluconate-6-C^{14} and gluconate-6-C^{14} the radioactivity was found in the methyl group of lactic acid. These are the expected results assuming the pathway is the same as that for glucose, where carbon dioxide arises from carbon-1 while the methyl group of lactic acid arises from carbon-6 (8).

TABLE I

C^{14}-DISTRIBUTION IN PRODUCTS FROM FERMENTATION OF LABELED 2-KETO-D-GLUCONATE, D-GLUCONATE, AND D-FRUCTOSE

		C^{14} as % of total in the products*				
		2-Ketogluconate		Gluconate		Fructose
Carbon No.	Product	-1-C^{14}	-6-C^{14}	-1-C^{14}	-6-C^{14}	-1,6-C^{14}
1	CO_2	96.5	1.4	98.8	0.4	49.3
	Acetic acid and ethanol					
2	—CH_3	0.1	1.5	<0.1	0.6	0.9
3	—COOH and —CH_2OH					
	Lactic acid					
4	—COOH	1.1	0.4	0.4	0.2	0.8
5	—CHOH—		0.1		<0.1	0.5
6	—CH_3		95.1		98.2	47.6

*The products from 2-ketogluconate were equimolar amounts of carbon dioxide, acetic acid, and lactic acid; from gluconate, equimolar amounts of carbon dioxide and lactic acid plus 0.5 molar amounts of ethanol and acetic acid; while one-third of the fructose was reduced to mannitol, the remainder was fermented to equimolar amounts of carbon dioxide and lactic acid plus 0.75 molar amount of ethanol and 0.25 molar amount of acetic acid (4).

DeMoss *et al.* have demonstrated the presence of glucose-6-phosphate dehydrogenase (10) and 6-phosphogluconate dehydrogenase (9) in cell-free extracts of *Leuconostoc mesenteroides*. Thus, it appears likely that the metabolism of glucose proceeds through glucose-6-phosphate to 6-phosphogluconate. However, the product resulting from the action of 6-phosphogluconate dehydrogenase on 6-phosphogluconate has not been identified and the succeeding metabolic steps are unknown.

Leuconostoc mesenteroides is reported to lack aldolase (8) and thus fructose diphosphate is not split and the Embden–Meyerhof glycolytic pathway cannot operate. Therefore the metabolism of fructose by this organism may involve a preliminary conversion of fructose to glucose or glucose-6-phosphate by a reversal of the glycolytic enzymes which are known to be present. The similarity of the products of fermentation from fructose and glucose, together with the radioactive distribution data given in Table I, support this view.

Mannitol was recovered from the culture liquor after the fermentation of fructose-1,6-C^{14} and recrystallized to a constant melting point (165° C.) and specific activity. The results are given in Table II and indicate that fructose is reduced to mannitol directly although the participation of phosphate esters cannot be disregarded.

TABLE II

COMPARISON OF SPECIFIC ACTIVITIES OF THE FERMENTED FRUCTOSE-1,6-C^{14} WITH THE RECOVERED MANNITOL

Compound	Specific activity (mμc. per mM. compound)
Fructose-1,6-C^{14}	107.5
Mannitol	102.9

The distribution of radioactivity among the products, as illustrated in Table I, demonstrates that *Leuconostoc mesenteroides* may be readily adapted to the biological degradation of 2-keto-D-gluconate, D-gluconate, and D-fructose.

Acknowledgment

The authors wish to thank Drs. A. C. Neish and A. S. Perlin for gifts of radioactive sugars, Mr. J. Dyck for the analyses of samples for radioactivity, and Mr. C. A. Matthews and Mr. J. H. Wasson for technical assistance.

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OBSERVATIONS ON THE METHIONINE NUTRITION OF STREPTOCOCCUS LACTIS[1]

I. HUSAIN[2] AND I. J. MCDONALD

Abstract

In a medium containing pyridoxal, a strain of *S. lactis* grew when arginine, histidine, isoleucine, leucine, phenylalanine, proline, valine, and either methionine, homocysteine, or cystathionine were supplied. Serine did not stimulate growth with homocysteine. In a medium with a mixture of 18 amino acids, pyridoxal was not required for growth; methionine was essential but homocysteine or cystathionine failed to replace methionine unless pyridoxal was present. These results indicated that conversion of homocysteine to methionine by this organism may involve vitamin B_6 but not serine.

Introduction

During the course of studies on the amino acid requirements of lactic streptococci, a strain of *Streptococcus lactis* (No. 829) was found to grow with a mixture of eight amino acids plus asparagine and glutamine (11). The amino acid mixture contained methionine for which the organism seemed to possess an absolute requirement since growth did not occur when it was not added to the medium.

Recently Kizer, Speck, and Aurand (12), in a study concerning the effect of methionine and methionine precursors on the growth of *S. lactis*, demonstrated the ability of this organism to manifest appreciable growth in the absence of methionine. Further, they observed that methionine could be replaced by a mixture of serine and homocysteine but not by homocysteine alone. On the basis of these data, they suggested that synthesis of methionine by this organism proceeded via cystathionine, homocysteine, and subsequent methylation. Later, they presented evidence (13) to show that phosphoserine was two to three times more effective than serine in promoting growth when homocysteine was substituted for methionine in the medium.

While studies on the synthesis of methionine in microorganisms have been numerous, the report of Kizer *et al.* (12) was the first account of its formation by *S. lactis*. In view of the fact that *S. lactis* 829 grew consistently in a limited number of amino acids and because it apparently was capable of utilizing homocysteine as well as methionine, it became of interest to study its methionine nutrition.

Materials and Methods

Streptococcus lactis 829, described previously (11), was maintained by weekly transfer in milk containing 0.5% glucose and 0.5% yeast extract.

[1]Manuscript received May 3, 1957.
Contribution from the Division of Applied Biology, National Research Laboratories, Ottawa 2, Canada.
Issued as N.R.C. No. 4394.
[2]N.R.C. Postdoctorate Fellow, 1956–57.

Composition of the double strength basal medium (Medium A) was the same as described previously (11). In experiments designed to test the effect of various components of the basal medium on growth of the organism in the presence of homocysteine or methionine, medium A was modified by omitting *p*-aminobenzoic acid, folic acid, vitamin B_{12}, thymidine, pyridoxamine dihydrochloride, leucovorin, and pantethine. The growth factors present in this modified medium (Medium B) were, therefore, thiamine, calcium pantothenate, riboflavin, pyridoxal, nicotinic acid, and biotin.

The double strength medium was adjusted to pH 6.5 and distributed in 5.0 ml. amounts. The amino acids L-arginine.HCl, L-histidine.HCl, DL-isoleucine, L-leucine, DL-phenylalanine, L-proline, DL-valine were combined in a single solution at such concentrations that 1.0 ml. supplied 400 μg. of DL- and 200 μg. of L-amino acid; hydrochlorides of L-arginine and L-histidine were used at the same concentration as the DL-acids. Other substances were added at concentrations indicated under appropriate tables. After all additions had been made, the volume in each tube was adjusted to 9.5 ml. with distilled water and the tubes were sterilized by autoclaving at 15 lb. pressure for 15 minutes. A Seitz filtered solution (0.5 ml.) containing 0.5 mg. of asparagine and 0.5 mg. of glutamine was then added to each tube containing the cold sterile medium.

Cells used as inocula were from second subcultures in tryptone–tryptose broth (Medium II (16)). After incubation at 30° C., the cells were centrifuged, washed three times, and diluted to 10 times their original volume in sterile 0.9% saline. An inoculum of 0.1 ml. of the suspension so obtained was used per 10 ml. of medium. After incubation at 30° C., growth was measured turbidimetrically at 660 mμ using a Coleman Junior spectrophotometer. The acid produced after 72 hours' incubation was titrated to the phenolphthalein end point and reported as ml. of 0.1 *N* NaOH per 10 ml. of culture corrected for the titration of the uninoculated control.

Methionine was assayed microbiologically by the procedure of Barton-Wright (2), using *Leuconostoc mesenteroides* P-60 (A.T.C.C. 8042) as the test organism and dehydrated methionine assay medium (Difco) as prescribed by Steele *et al.* (21). Methionine was estimated chemically by the method of McCarthy and Sullivan (15). In chromatographic analyses of cell hydrolyzates, sulphydryl groups were protected by N-ethyl maleimide as recommended by Hanes *et al.* (9). Dried papers were sprayed with platinic iodide reagent as modified by Toennies and Kolb (22).

Results

A mixture of amino acids containing arginine, histidine, isoleucine, leucine, methionine, phenylalanine, proline, and valine supported growth of the organism in an otherwise nutritionally complete, chemically defined medium (Medium A). Growth in the presence of this amino acid mixture always appeared within 24 to 36 hours and was about 70% of that obtained in a medium containing 18 amino acids (10). When methionine was omitted from

TABLE I

GROWTH RESPONSE OF *S. lactis* 829 TO DL-METHIONINE, DL-HOMOCYSTEINE, L-CYSTEINE HCl, AND DL-ALLO(+) CYSTATHIONINE (30° C.)

Addition to medium*	% light transmitted			Acid formed†
	24 hr.	48 hr.	72 hr.	72 hr.
None	100	100	99	0.1
DL-Methionine‡	66	60	51	3.8
DL-Homocysteine‡	64	63	55	3.8
DL-Homocysteine‡ plus DL-serine‡	65	62	54	3.8
DL-Allo(+) cystathionine‡	59	57	49	3.9
L-Cysteine HCl‡	99	97	97	0.3
L-Cysteine HCl‡ plus DL-homoserine‡	78	64	52	3.6

*Medium A (see text) plus L-arginine HCl, L-histidine HCl, DL-isoleucine, L-leucine, DL-phenylalanine, L-proline, and DL-valine.
†Ml. 0.1 *N* NaOH/10 ml. culture.
‡1 ml. *M*/375 solution per 10 ml. final medium.

the medium, growth did not occur. In preliminary experiments with media containing amino acids at the concentrations used by Kizer *et al.* (12), growth was not reduced by omission of methionine. However, when the concentrations of amino acids were reduced to one-fifth that used by Kizer *et al.* (12), methionine was absolutely essential. The apparent discrepancy in the two observations was traced to contamination of leucine with about 16% methionine. In later experiments with a pure sample of leucine, methionine was essential regardless of the concentration of amino acids in the medium.

In medium A, which contained pyridoxal, *S. lactis* 829 grew as well with homocysteine or cystathionine as it did with equimolar concentrations of methionine (Table I). Cysteine, however, was unable to support growth unless homoserine was present in addition. Serine with homocysteine did not improve growth nor did serine stimulate growth when suboptimal amounts of homocysteine were used. Other sulphur containing compounds and reducing agents such as sodium thioglycolate, sodium thiosulphate, mercaptosuccinic acid or ascorbic acid were unable to support growth when substituted for methionine in the medium.

The ability of carbon dioxide to replace serine in the presence of glycine for growth of *Leuconostoc mesenteroides*, and to replace methionine in the presence of homocysteine for growth of *Streptobacterium plantarum* has been reported (5, 14). In the present study, the growth of *S. lactis* was least in an atmosphere of nitrogen, but increased with the carbon dioxide tension. However, at any carbon dioxide tension, the same amount of growth was obtained with homocysteine as with methionine. It would appear, therefore, that the ability of *S. lactis* to grow with homocysteine alone was not caused by carbon dioxide, unless, of course, metabolic carbon dioxide was involved. The amount of metabolic carbon dioxide, however, should not be great, since *S. lactis* is a homofermentative organism.

In a modified medium (Medium B), growth of the organism with homocysteine was less than that obtained with methionine (Table II). Additions

of folic acid, vitamin B_{12}, thymidine, leucovorin, or pantethine in the presence of homocysteine did not improve the growth but addition of *p*-aminobenzoic acid stimulated growth so that it was the same with homocysteine as with methionine.

In medium B, with eight amino acids including methionine, pyridoxal was found to be essential for growth of *S. lactis* 829. However, pyridoxal was not required for growth if a mixture containing 18 amino acids, including methionine, was used (Table III). In this medium, without pyridoxal, cystathionine was unable to support growth, and growth with homocysteine was only about 35% of that obtained with methionine. When pyridoxal was present, however, homocysteine, cystathionine, or methionine gave the same amount of growth.

TABLE II

EFFECT OF VARIOUS COMPONENTS OF THE BASAL MEDIUM ON GROWTH OF *S. lactis* 829 IN PRESENCE OF DL-HOMOCYSTEINE OR DL-METHIONINE (72 HR. AT 30° C.)

Medium	% light transmitted	
	DL-Homocysteine‡	DL-Methionine‡
A*	62	59
B†	74	60
B plus folic acid	77	59
B plus vitamin B_{12}	74	60
B plus thymidine	77	61
B plus leucovorin	75	59
B plus pantethine	76	61
B plus *p*-aminobenzoic acid	60	59

*Medium A (see text and footnote to Table I).
†Medium B (see text). Amino acids used were the same as with Medium A.
‡See footnote to Table I.

TABLE III

EFFECT OF PYRIDOXAL ON GROWTH OF *S. lactis* 829 IN PRESENCE OF DL-HOMOCYSTEINE, DL-ALLO(+) CYSTATHIONINE, OR DL-METHIONINE (30° C.)

Addition to medium*	Pyridoxal	% light transmitted			Acid formed†
		24 hr.	48 hr.	72 hr.	72 hr.
None	Absent	100	100	100	0.1
DL-Homocysteine‡	"	85	81	79	1.3
DL-Methionine‡	"	63	56	53	3.7
DL-Allo(+) cystathionine‡	"	100	100	100	0.2
None	Present	100	99	99	0.1
DL-Homocysteine‡	"	50	44	37	4.1
DL-Methionine‡	"	48	42	36	4.6
DL-Allo(+) cystathionine‡	"	44	40	34	4.8

*Medium B (see text) without pyridoxal. Seventeen amino acids used instead of seven amino acids and *p*-aminobenzoic acid included in the medium.
†, ‡ See footnotes to Table I.

Cells grown with cystathionine or homocysteine in place of methionine were hydrolyzed and the hydrolyzates shown to contain methionine by microbiological assay and by paper chromatography. However, preliminary experiments to demonstrate conversion of homocysteine to methionine by washed cell suspensions of the organism have so far been unsuccessful.

Discussion

The strain of *S. lactis* used in the present studies required methionine for growth, which agrees with reports of other workers (1, 17) that lactic streptococci do not grow in the absence of methionine. A study of the minimum amino acid requirements for growth of lactic streptococci (11) also indicated that methionine was essential. The requirement for methionine could not be demonstrated, however, if the leucine used in the medium was contaminated with methionine, an occurrence that is apparently not uncommon (15). The findings of Kizer *et al.* (12) that eight strains of *S. lactis* grew appreciably when methionine was not added to the medium do not agree with other reports (1, 11, 17). These differences probably were due to the use of different strains although the possibility of leucine contaminated with methionine cannot be overlooked.

The methionine requirement of *S. lactis* 829 in a medium containing pyridoxal and a minimal mixture of seven amino acids was satisfied by either cystathionine or homocysteine. This suggested that methionine was synthesized by the organism, a possibility that was strengthened by the detection of methionine in hydrolyzates of cells grown in the presence of cystathionine or homocysteine. Since the minimal amino acid mixture did not contain serine, this amino acid apparently was not concerned in the conversion of homocysteine to methionine. This conclusion was supported by the evidence that serine did not stimulate growth when suboptimal amounts of homocysteine were used and that in a medium containing 18 amino acids, including serine, homocysteine did not fully replace methionine (unless vitamin B_6 was also present as discussed below). The pathway of methionine synthesis by this bacterium, therefore, seems to differ from that reported for other organisms. Thus Kizer *et al.* (12) found that several other strains of *S. lactis* required serine for growth in the presence of homocysteine and concluded that methionine was synthesized from homocysteine through methylation by the β-carbon of serine. Studies with mutants of *Escherichia coli* (7) also have resulted in strong evidence for serine as the donor of the one carbon unit.

The biosynthesis of methionine has been shown by several workers to involve vitamin B_{12}, *p*-aminobenzoic acid, and folic acid. Gibson and Woods (8) noted that both vitamin B_{12} and *p*-aminobenzoic acid were required for the conversion of homocysteine to methionine and the hypothesis suggested by Shive (18) that a coenzyme form of folic acid is concerned in the metabolic transfer of single carbon residue is now generally accepted. In the present study, *p*-aminobenzoic acid appeared to be active in the conversion while folic acid and vitamin B_{12} were not. However, the organism may have stored or

synthesized sufficient quantity of these latter two factors to allow methionine formation, a possibility suggested by the observation that growth of the organism with methionine was not affected by the omission of these substances from the medium.

Although homocysteine could replace methionine in a medium with a minimal amino acid mixture, it was only about 35% effective in a medium with a complete amino acid mixture when pyridoxal was absent. In the presence of pyridoxal, however, methionine and homocysteine were equivalent regardless of the amino acid mixture. Thus it appears that pyridoxal was essential for the conversion of homocysteine to methionine. The partial replacement of methionine by homocysteine in the absence of pyridoxal may have been due to endogenous pyridoxal synthesis by the organism. Nutritional studies (1, 17) tend to support this assumption. *S. lactis* appeared to possess a limited ability to synthesize vitamin B_6 because all cultures could be subcultured repeatedly in a pyridoxal-free medium although the turbidity developed was only half maximum (1, 17). The inability of cystathionine to support growth, in the absence of pyridoxal, also may have been due to the limited amount of vitamin B_6 synthesized by the organism. The enzyme responsible for cleavage of cystathionine is known to be B_6 dependent (3, 19) and if B_6 is required for conversion of homocysteine to methionine, the amount of the vitamin synthesized may have been insufficient to activate both enzymes (4, 19).

In the light of these observations, it is tempting to suggest that pyridoxal, possibly through its phosphorylated form, is actively involved in the conversion of homocysteine to methionine. It has been asserted that one of the primary roles of vitamin B_6 in metabolism is to catalyze reactions involved in the synthesis of amino acids (19) but it does not seem to have been implicated specifically in methionine biosynthesis (20). However, cell-free preparations of *E. coli* synthesized greater amounts of methionine from homocysteine and serine when pyridoxal phosphate was present than when it was absent (6). It has been shown by several workers that the amount of vitamin B_6 required for growth of many lactic acid bacteria depends markedly upon the number and type of amino acids in the medium. It was not surprising, therefore, that *S. lactis* 829 failed to grow without pyridoxal when a limited number of amino acids were used in the medium.

While it is not possible with the data presented to propose any definite mechanism for synthesis of methionine from homocysteine by this organism, the results do at least suggest that vitamin B_6 has a role in this conversion and that serine is not involved in the scheme.

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NUTRITION AND METABOLISM OF MARINE BACTERIA

VI. QUANTITATIVE REQUIREMENTS FOR HALIDES, MAGNESIUM, CALCIUM, AND IRON[1]

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Abstract

A study has been made of the specificity of the requirement of some marine bacteria for halides, magnesium, calcium, and iron and of their quantitative requirements for these ions in the presence and absence of related ions.

All of the organisms investigated either required or responded to the addition of Cl^- to the medium. Br^- could replace Cl^- but somewhat less readily, while I^- was toxic. For organism B-16, which required the addition of Mg^{++} but not Ca^{++} to the medium, the amount of Mg^{++} required for maximum growth varied with the level of Ca^{++} present. At low levels, a marked sparing action of Ca^{++} was evident, while at higher concentrations of Ca^{++} some antagonism between the ions was detected. Sr^{++} also spared the Mg^{++} requirement of this organism. For B-9, which required both Mg^{++} and Ca^{++}, Mg^{++} could be shown to spare the need for Ca^{++}.

An absolute requirement of one organism for Fe^{++} was demonstrated. Ni^{++} and Co^{++} showed no sparing action and proved to be toxic at a level at which Fe^{++} produced an optimum growth response.

It has been shown that an appropriate mixture of inorganic ions can replace sea water in a chemically defined medium for the growth of a number of marine bacteria. Qualitative requirements of several of the organisms for various ions have been established, and the relation of Na^+ and K^+ to their growth considered in some detail (7, 8). This report concerns an investigation of the specificity of the requirement of the organisms for halides, magnesium, calcium, and iron, and of their quantitative requirements for these ions in the presence and absence of related ions.

Methods

Organisms

The sources of the organisms and the general methods used in their study have been described (6). The four organisms used in this study have been classified as follows: B-9, a *Flavobacterium*; B-10 and B-20, *Pseudomonas*; B-16, either a *Pseudomonas* or a *Spirillum*. In the case of three of the organisms, this represents a change in their classification over that previously published.

Media and Assay Procedures

The precautions taken in the preparation of the basal medium and the assay procedures used were the same as those applied previously (7). The basal medium contained (in amounts per 10 ml. of final medium): glucose, 30 mg.; DL-α-alanine, 24 mg.; DL-aspartic acid, 9.6 mg.; glutamic acid, 12 mg.; $(NH_4)_2SO_4$, 8.7 mg.; $(NH_4)_2HPO_4$, 1.3 mg.; NaCl, 127 mg.;

[1]Manuscript received March 28, 1957.
Contribution from The Fisheries Research Board of Canada, Technological Station, Vancouver, B.C.

$MgCl_2$, 25.3 mg.; $CaCl_2$, 5.5 mg.; $FeSO_4.(NH_4)_2SO_4$, 250.7 μg. Unless otherwise stated in the text, the inoculum medium was composed of (in amounts per 10 ml. of final medium): Bacto nutrient broth, 80 mg.; yeast extract, 50 mg. plus the same salts at the same levels that were added to the basal medium.

Salts Used

In all cases, reagent grade salts were used without further purification. Analyses were made for contaminating traces of Mg^{++} in the Ca^{++} salts and of Ca^{++} in the Mg^{++} salts, using a flame photometer attachment for a Beckman DU spectrophotometer equipped with a photomultiplier. Mg^{++} estimations were made at two wave-lengths, 285.2 and 371 mμ, and Ca^{++} at 554 mμ (3).

Results

Response to Halides

When chloride salts were replaced by their corresponding sulphates or nitrates in the preparation of the basal medium one marine bacterium failed to grow and several grew more slowly unless chloride ion was added back (7).

The effect of halides on the growth of four of the marine bacteria studied previously was considered in more detail in this investigation. The response of organism B-9 to Cl^-, Br^-, and I^- is shown in Fig. 1. In this experiment, the chloride salts present in the basal medium were replaced by their corresponding sulphates except in the case of Ca^{++}, which was added as the nitrate. The halides were added as their Na^+ salts. It is immediately evident that,

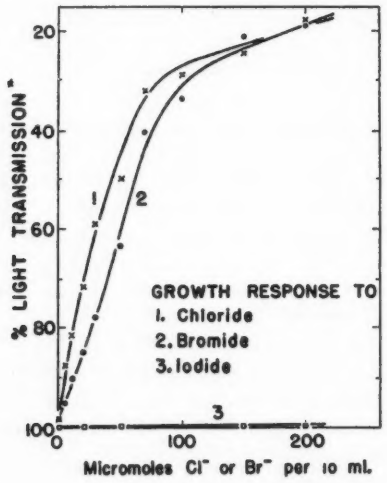

FIG. 1. The response of marine bacterium B-9 to halides.
*Evelyn colorimeter readings, 660 mμ filter, uninoculated flask = 100. Incubation time 112 hr.

mole for mole, Br^- was almost as effective as Cl^-, while I^- was without activity in promoting growth of the organism. The amount of Cl^- or Br^- required for growth varied with the incubation period. For maximum growth in the shortest time (about 72 hours for this organism) 1 millimole of Cl^- or Br^- was required for 10 ml. of medium. After sufficiently long incubation periods (10 days) appreciable growth occurred with 10 micromoles of added halide. Organism B-20 resembled B-9 in failing to grow without added halide even on long incubation, while B-10 grew to a limited extent under these conditions in 6 days. In all cases Br^- but not I^- could replace Cl^- almost mole for mole. I^- proved to be inhibitory.

In the case of organism B-16, only the rate of growth could be shown to be reduced by lack of added halide and then only for a brief period in the life of the culture. The response of the organism to halides is shown in Table I after two incubation periods. The amount required for maximum stimulation of growth (2 millimoles per 10 ml.) is about two-thirds of the Cl^- level in sea water.

TABLE I

GROWTH RESPONSE OF ORGANISM B-16 TO Cl^-, Br^-, AND I^-

Anion added, μM./10 ml.	Cl^-		Br^-		I^-	
	Incubation time (hr.)					
	41	66	41	66	41	66
	% incident light transmitted*					
0	90	29	91	34	95	34
10	84	33	82	39	82	30
100	80	41	79	45	96	44
500	56	43	61	36	100	99
1000	38	34	36	35	100	100
2000	29	22	35	24	100	100

*Evelyn colorimeter readings, 660 mμ filter, uninoculated flask = 100.

Response to Mg^{++} and Ca^{++}

All of the marine bacteria investigated previously failed to grow or grew to only a limited extent if Mg^{++} was omitted from the medium. Lack of added Ca^{++} prevented the growth of one organism and reduced the rate of growth of another (7).

When the quantitative requirements of the organisms for Mg^{++} and Ca^{++} were investigated it was found that the levels needed for maximum growth varied markedly, depending upon the amounts of other alkaline earth metal ions present in the medium. This is illustrated in Table II where the response of organism B-16 to Mg^{++} is shown at different levels of Ca^{++} in a basal medium from which both Mg^{++} and Ca^{++} salts had been omitted. In the absence of added Ca^{++}, the Mg^{++} level required for growth was exceptionally high. The addition of 25 micromoles of Ca^{++} reduced very remarkably, but could not replace the requirement for Mg^{++}. Sr^{++} had a

TABLE II

RESPONSE OF ORGANISM B-16 TO Mg^{++} IN THE PRESENCE AND ABSENCE OF Ca^{++}

Mg^{++}, μM./10 ml.	Ca^{++} added (μM. per 10 ml.)		
	0	25	500
	% light transmission*		
0	100	90	100
0.004	—	80	100
0.02	—	64	100
0.2	—	42	98
0.4	100	29	57
2.0	98	22	25
4.0	97	18	26
40.0	22	18	19
80.0	16	16	18

*See Table I. Incubation time 70 hours; Mg^{++} and Ca^{++} added as their chloride salts.

TABLE III

RESPONSE OF ORGANISM B-9 TO Ca^{++} AND THE SPARING ACTION OF Mg^{++}

Ca^{++}, μM./10 ml.	Mg^{++} added (μM./10 ml.)	
	20	200
	% light transmission*	
0	100	100
1.0	100	100
2.0	100	93
3.0	100	32
5.0	100	37
7.0	95	22
10.0	86	18
20.0	20	18

*See Table I. Incubation time 70 hours; Mg^{++} and Ca^{++} added as their chloride salts.

similar sparing action. Supplementing the medium with 500 micromoles of Ca^{++}, on the other hand, increased the Mg^{++} requirement over that existing when only 25 micromoles of Ca^{++} were present.

The reverse effect, the capacity of Mg^{++} to spare the requirement of an organism for Ca^{++}, is shown in Table III with organism B-9. This organism required the addition of both Mg^{++} and Ca^{++} to the medium for growth. If a small amount of Mg^{++} was added to the medium, much more Ca^{++} was required to support growth than if a higher level of Mg^{++} was present. If the sparing action of Mg^{++} shown were due to the ability of the Mg^{++} salt to introduce contaminating traces of Ca^{++}, it can be calculated that the

Mg^{++} salt would have had to contain at least 6.6% Ca^{++} to account for the effect. Direct analyses by flame spectrophotometry showed the Mg^{++} salt to contain only 0.019% Ca^{++}. Similarly, the sparing action of Ca^{++} on the response to Mg^{++} shown in Table II could not be accounted for by Mg^{++} added with the Ca^{++} since only 0.071% Mg^{++} was found in the Ca^{++} salt.

Be^{++} and Mn^{++} salts showed no capacity to replace or spare the requirement of any of the organisms tested for Mg^{++} or Ca^{++} and were found to be toxic at low concentrations.

Response to Fe^{++}

A supplement of Fe^{++} in a sea-water medium has been shown to increase the rate and extent of growth of all the marine bacteria investigated in this laboratory (7). Though complete lack of growth in the absence of added Fe^{++} could not be demonstrated at that time, a relatively complex medium apt to contain contaminating traces of Fe^{++} was used.

In an effort to demonstrate a more absolute requirement for Fe^{++}, one of the organisms, B-16, was chosen which would grow well on a medium simpler in composition than the basal. This medium contained 0.7% succinate in place of the glucose and amino acids. The growth response of the organism to Fe^{++} in this medium is shown in Fig. 2. Essentially no growth occurred in the absence of added Fe^{++} even after a 9 day incubation period, while maximum growth resulted in only 48 hours on the addition of approximately 0.3 micromoles of iron per flask. An almost identical response was obtained if a ferric salt was used to supply iron. Ni^{++} and Co^{++} showed no capacity to replace or spare the Fe^{++} requirement and were toxic when tested at the 0.2 micromole level.

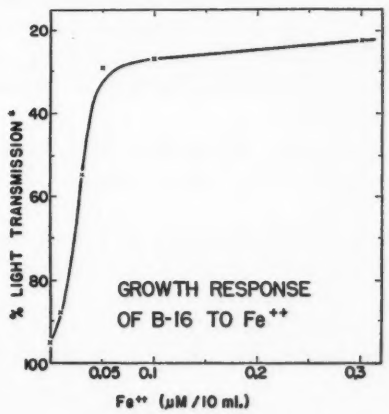

FIG. 2. Growth response of marine bacterium B-16 to Fe^{++} in a simplified synthetic medium.
*See Fig. 1. Incubation time 48 hours; Fe^{++} added as $FeSO_4.(NH_4)_2SO_4$.

Discussion

Although the importance of Cl^- in biological systems has been suspected for a considerable time, only recently has evidence been obtained that at least some cells have a requirement for this anion for growth. Broyer *et al.* (2) showed that tomato plants require Cl^- and that this requirement could be satisfied at least partially by much higher levels of Br^- while I^- proved to be toxic. There is an obvious similarity between these findings and those reported here for marine bacteria. Eagle (4) found that two types of mammalian cells in tissue culture needed Cl^- for growth, the optimum level being very similar to that required by the marine bacteria. The only other report of a requirement of bacteria for halides has been made by Brown and Gibbons (1) for the halophile *P. salinaria.*

It is of interest that so much more Cl^- was required by the marine bacteria for maximum rate of growth than was needed to permit growth after a sufficiently long incubation period. Even for organism B-16, for which no absolute requirement for Cl^- could be demonstrated, the level for maximum rate of growth was of the same order of magnitude as that for the other organisms. The response of these bacteria to halides and to Na^+ is similar in respect not only to the effect of the ions on rate and extent of growth but also to the amounts of them required for maximum rate of growth. It thus seems not unlikely that the function of Na^+ and halide may be closely related in the metabolism of marine bacteria.

The capacity of Ca^{++} to reduce so markedly the requirement of the organisms for Mg^{++} and vice versa suggests that for some but not all of the functions of these ions in the metabolism of the organisms, the two ions can be used interchangeably. The antagonism evident between the two ions at the higher concentration of Ca^{++} for organism B-16 is indicative of a situation where Mg^{++} is required specifically in a particular function and Ca^{++} can act at this site as an antimetabolite. These observations are very similar to the relationships between Mn^{++} and related ions previously recorded for lactic acid bacteria (5).

The level of iron required in the medium for maximum growth of organism B-16 is very nearly the same as that recorded for *E. coli* by Young, Begg, and Pentz (9).

Since there have been many reports of requirements of land forms of bacteria for Mg^{++}, Ca^{++}, and Fe^{++}, of the data reported here, only the response to halides appears to be unique to marine bacteria. This response to halides, like the previously reported requirement for Na^+ (7, 8), may prove to be a feature distinguishing marine bacteria from their terrestrial counterparts.

Acknowledgment

We are indebted to Dr. Einar Leifson, Loyola University, Chicago, for reviewing our classification of these organisms.

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CELL-WALL-SPLITTING ENZYMES OF PUCCINIA GRAMINIS VAR. TRITICI[1]

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Abstract

Evidence is given for the existence of a hemicellulase and a cellulase in uredospores of *Puccinia graminis* var. *tritici*. Pectinase is also present and is shown to be an adaptive enzyme. The pH and temperature optima of these three cell-wall-splitting enzymes have been determined by means of viscosimetric measurements. Preliminary investigations using the mannogalactan of *Ceratonia siliqua* L. as a substrate for hemicellulase and carboxymethylcellulose as a substrate for cellulase indicate that degradation with the enzymes of ungerminated rust involves attack on internal linkages of the substrate molecule, giving a rapid decrease in viscosity with slow production of free reducing groups. The influence of heavy metal salts, reducing agents, and iodoacetic acid was examined.

Introduction

Previous studies in this laboratory (31, 32) on the enzymatic constitution of uredospores of wheat stem rust have indicated the presence of the enzymes of the glucose-6-phosphate oxidative pathway as well as enzymes that can oxidize an external source of glutamic acid and other amino acids. The present investigation was conducted to determine if the uredospores contained or produced on germination enzymes capable of hydrolyzing plant polysaccharides. Such enzymes probably play an important role both during the germination of the rust spores and during infection of the stem and leaf of the host.

Since the first experiments with enzymes that attack components of the cell wall were made by Biedermann and Moritz (2) and Effront (6), a variety of substrates have been used by different investigators. Cellulase activity has been studied on cellulose, filter paper, tunicine, and bacterial cellulose (10, 11, 17, 18) as well as β-glucosan of barley (24, 25) and hydroxyethylcellulose (30, 40). Freeman *et al.* (9), Holden and Tracey (14), Reese *et al.* (29), Jermyn (16), and Van Sumere (35, 36) measured the cellulase activity by employing sodium carboxymethylcellulose. Sodium carboxymethylcellulose (CMC) is a water-soluble substrate which permits attack on the β-glucosidic linkages of the cellulose-like material in a homogeneous system. The degree of substitution (DS) influences strongly the water solubility and enzymic degradation of the CMC, a higher DS improving the solubility but decreasing the enzyme action (20, 36). The breakdown of CMC can thus be seen as a measurement

[1]Manuscript received March 25, 1957.
Contribution from the National Research Council of Canada, Prairie Regional Laboratory, Saskatoon, Saskatchewan.
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of the presence of an enzyme that is capable of hydrolyzing polymeric β-glucosidic linkages. The term "cellulase", as generally used, refers to the ability to cleave the initial cross-linkages in native cellulose, while the enzyme(s) responsible for splitting the β-glucosidic linkages is usually called "C_x" enzyme.

A still wider variety of substrates has been used to determine hemicellulase activity. Effront (6) followed the activity of the seed extract of *Ceratonia siliqua* L. on the endosperm of the same seed. Mannogalactans of carube and alfalfa seed (3, 4, 5), sugar beet araban (7, 8), tragacanth gum (12), xylans of barley endosperm (21), and a variety of wood materials have been used (10, 11, 23).

More recently Preece and Hobkirk (27), using a highly purified rye araboxylan, studied the hemicellulase action of barley. Massart and Van Sumere (22), using the same enzyme source, followed the degradation of the mannogalactan of carube and barley hemicelluloses. In this investigation the activity of the rust hemicellulase was compared on several substrates.

The pectic enzyme, polygalacturonase (pectinase), splits the α-1,4-glycosidic linkages of the anhydrogalactosidic acid chain of pectin. Pectic acid appears to be the true substrate of pectin polygalacturonase since the velocity of the hydrolysis increases roughly in proportion to the decrease in extent of esterification (15, 39). In our experiments we made use of Pectin N.F. with a methoxy content of 11.65%.

Materials and Methods

Enzymes

Crude enzyme preparations were obtained from two lots of spores, one collected in 1955 (old spores) and another lot collected in 1956 (new spores).

A 7.2 g. quantity of rust uredospores (*Puccinia graminis* var. *tritici* race 15B) were ground for 3 hours in a ball mill with 40 ml. 0.6% NaCl solution. The cell debris was separated by centrifugation (2000 r.p.m.). The proteins in the crude extract were precipitated with ammonium sulphate and redissolved in water to a volume half that of the crude extract. Concentration of the enzymes by precipitation with acetone or alcohol resulted in a 50 to 80% loss. In some cases the rust was first germinated on agar. Since the rust spores possessed an adaptive pectinase, the spores were germinated on agar with added pectin to obtain the pectin enzymes for study. The germinated spores were then collected and ground as before. The hydrolytic enzymes in the concentrated extracts appeared to be fairly stable and could be stored for a week or more at 2°–4° C.

Substrates

Cellulase activity, that is, the ability of the uredospore extracts to cleave the β-glucosidic linkages ("C_x" enzyme) was determined on sodium carboxymethylcellulose (CMC) (Hercules Powder Co., type 70, high viscosity) (9, 20, 35, 36). In addition a 0.5% solution of the β-glucosan from barley prepared

by the method of Preece and MacKenzie (28) was used. This latter preparation contains both β-glucosidic 1,4- and β-glucosidic 1,3-linkages (1) thus resembling lichenine more than cellulose. The carboxymethyl substrate was prepared by treating 1 g. CMC with 50 ml. water to allow swelling to take place. After 50 ml. buffer and another 50 ml. water were added to give a 0.75% dilution, the mixture was thoroughly homogenized in a Waring blendor.

Hemicellulase activity was determined on the mannogalactan of *Ceratonia siliqua* L. This polysaccharide contains ± 20% D-galactose and 80% D-mannose. According to Hirst and Jones (13), the main chain consists of D-mannose units linked through β-glucosidic 1,4-linkages, while the D-galactose side groups are attached to this mannose chain through β-glucosidic 1.6 bonds. The substrate was prepared by refluxing 5 g. mannogalactan with 500 ml. of water. After 30 minutes the mixture was homogenized with another 250 ml. water in a Waring blendor and the undissolved residue removed by centrifugation.

In addition three other substrates were used to determine hemicellulase activity. Barley hemicelluloses were prepared following the methods of Preece *et al.* (26) and *Tubera salep* mannan following the procedure of Klages and Niemann (19). Both substrates were used in 0.5% concentrations. The salep mannan molecule is believed to contain an open chain composed of 60 mannopyranose units which are β-glucosidic linked. The chemical structure of the barley hemicelluloses is not yet well understood.

Linseed mucilages were prepared by shaking 100 g. flax seed with 500 ml. water for 12 hours. After the mixture was filtered through a Büchner filter, two volumes of alcohol were added and the precipitated mucilage centrifuged. The white residue was again dissolved in water and reprecipitated with alcohol; the mucilage was washed with 95% ethanol and ether and dried under vacuum. A 0.5% aqueous solution was used as a substrate in the experiments reported here.

Because cell wall material of resistant and non-resistant wheat strains might differ in their susceptibility to the action of rust enzymes, we prepared hemicelluloses of Khapli (resistant) and Little Club (non-resistant) wheats. Both wheats were grown for about three weeks in the greenhouse before leaves and stems were collected and dried under vacuum. The materials were then ground with a Wiley mill to pass a 60-mesh screen. After extraction with a 1:2 alcohol–benzene solution and with hot water (following the standard Tappi procedure as applied to wood (34)), the residue was dried and the water extracts combined. A gum fraction was precipitated by the addition of ethanol to the filtered water extracts. Twenty-five grams dried residue were then successively extracted with 250 ml. 1 *N* NaOH. The extracts were clarified and the hemicellulose fraction obtained by precipitation with ethanol. On acid hydrolysis, the gum and hemicellulose fractions yielded large amounts of xylose, some arabinose, and glucose. Equal amounts of gum were extracted from both varieties but 20% more hemicellulose was obtained from Khapli wheat than from Little Club. Because of the lower viscosity of these wheat hemicelluloses, 2.5% solutions were used in our experiments.

Viscosimetric Measurements

The action of the "C_x" enzyme, hemicellulase, and pectin polygalacturonase were followed viscosimetrically using the Ostwald viscosimeter. The enzyme activity was calculated by means of the following formula

$$V_R = [(T_0 - T_t)/T_t] \times 100 .$$

In this formula V_R represents the relative viscosity to the flow rate in seconds of the blank (T_0) and the flow rate of the reaction mixture (T_t) after a time interval of t. V_R plotted against the reaction time shows exponential activity curves. The increase of the relative viscosity as a function of time is thus a measurement of the enzyme activity. The measurements were made as follows: 6 ml. of substrate and 2 ml. of buffer were mixed and 1 ml. of the enzyme preparation added. The viscosity (T_t) was then measured at intervals. The blank (T_0) was taken under the same conditions except that boiled enzyme was used.

Reductometric Measurements

The production of reducing groups during the enzymatic degradation of the substrates was determined with the dinitrosalicylic acid reagent of Sumner (33). Four milliliters of substrate–buffer mixture (for the appropriate buffer see under pH optimum) and 2 ml. enzyme were mixed and incubated at 40° C. for a 1- to 5-hour period. Three-milliliter aliquots were withdrawn for analysis and the intensity of the color read in a Coleman Junior Spectrophotometer. The data were corrected for the reducing substances present in the appropriate controls.

Results

pH and Temperature Optima

The enzyme activity of extracts from rust spores (old spores) germinated on agar was measured at different pH values on CMC, on the mannogalactan of *Ceratonia siliqua* L., and on pectin. A $M/10$ disodium citrate – 1/10 N HCl buffer was used for pH 2 and 3, a $M/5$ acetate buffer for the pH range 3 to 5, and a $M/7.5$ phosphate buffer for the pH range 5 to 8. Pectin polygalacturonase was not detectable in these extracts and proved to be a strongly adaptive enzyme. To obtain high polygalacturonase activity it was necessary to germinate rust spores on agar containing pectin.

The enzyme activity followed the Schütz rule, i.e. hydrolysis of the substrate was proportional to the square root of the reaction time. The pH optimum for the "C_x" enzyme, hemicellulase, and polygalacturonase from germinated rust spores are located at 5.5, 5.5, and 4.5, respectively (Fig. 1).

The temperature optimum for the hemicellulase and pectinase were found to be at 40° C. while the "C_x" enzyme proved to be slightly more active at 50° C. (Table I).

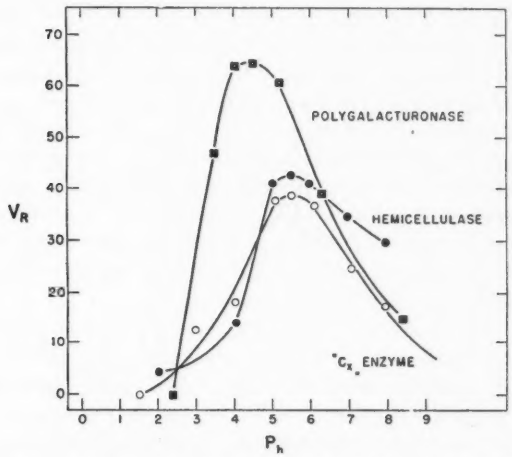

FIG. 1. The pH optima for the "C_x" enzyme, hemicellulase, and polygalacturonase obtained from 1-year-old germinated uredospores when allowed to act on their respective substrates for 15 minutes.

TABLE I

TEMPERATURE OPTIMUM OF THE CELL-WALL-SPLITTING RUST ENZYMES

Relative viscosity (V_R) after 15 minutes

Enzyme	Substrate	Temperature		
		30° C.	40° C.	50° C.
"C_x" enzyme	CMC	27.5	28.5	35.0
Hemicellulase	Mannogalactan	16.5	44.5	25.5
Polygalacturonase	Pectin	53.0	61.5	55.0

TABLE II

EFFECTS OF AGE OF UREDOSPORES ON THE PRODUCTION OF CELL-WALL-SPLITTING ENZYMES

Relative viscosity (V_R) after 15 minutes

Enzyme	Old spores*	Fresh spores†	Germinated fresh spores
"C_x" enzyme	38.0	16.0	31.5
Hemicellulase	51.0	19.5	28.5
Polygalacturonase	0.0	0.0	65.5

*Spores collected in the fall of 1955.
†Spores collected in 1956.

Comparison between the Cell-Wall-Splitting Activity of Germinated and Ungerminated Rust Spores

The activities of 1-year-old rust spores, fresh spores, and germinated fresh spores were compared under optimal conditions (Table II).

Activity of the Extract From Old Rust Spores on Different Substrates

The ability of an extract from old rust spores to degrade the mannogalactan of *Ceratonia siliqua* L. (carube), CMC, wheat plant hemicelluloses, barley β-glucosan, *Tubera salep* mannan, barley hemicelluloses (S_2 fraction, Kenya barley), and linseed mucilage and the ability of extracts from germinated spores to degrade pectin was determined (Fig. 2). The enzyme activity was measured viscosimetrically at pH 4.5 and 40° C. in the case of pectin polygalacturonase and at pH 6 and 50° C. for the "C_x" enzyme. The activity on linseed (pH 5.5) was determined without the use of buffer as the buffers precipitated the substrate (36). The activity on the other substrates was followed at pH 6 and 40° C.

Although the viscosity of the substrates decreased quickly, only a slow increase in reducing power could be noted (Fig. 3). When the desalted and concentrated reaction mixtures with CMC were chromatographed, the presence of a sugar with the same R_F as glucose was found. In the enzyme hydrolyzates of carube mannogalactan, galactose and a little mannose were detected. Reaction mixtures of wheat hemicelluloses contained xylose, xylobiose, xylotriose, and an unidentified higher polymer of xylose. Glucose or cellobiose were not found, although the hydrolyzed wheat hemicelluloses

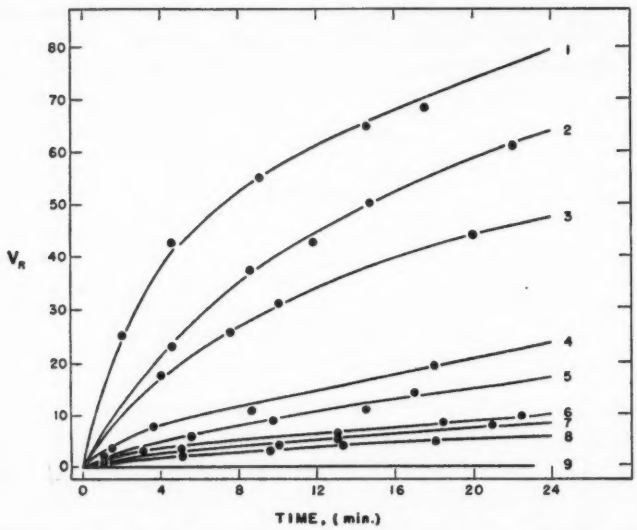

FIG. 2. Comparison of the activity of cell-wall-splitting enzymes of old rust spores on different substrates.

1. Pectin.
2. *Ceratonia siliqua* L. mannogalactan.
3. CMC.
4. Non-resistant wheat hemicellulose (Little Club).
5. Resistant wheat hemicellulose (Khapli).
6. Barley β-glucosan.
7. *Tubera salep* mannan.
8. Barley hemicellulose (S_1 fraction from Kenya barley).
9. Linseed mucilage.

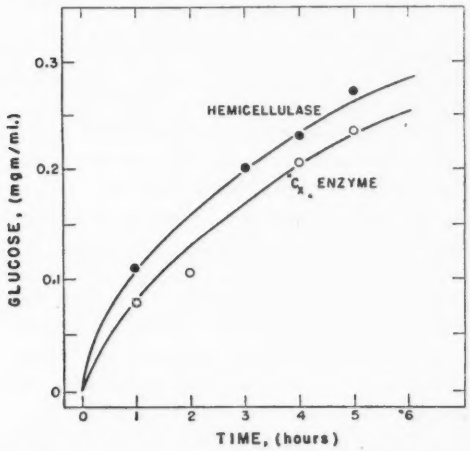

FIG. 3. Production of reducing groups during enzymatic degradation of CMC and carube mannogalactan.

contained arabinose and glucose in addition to the predominant amount of xylose. As expected, degradation of pectin resulted in production of D-galacturonic acid along with smaller amounts of D-galactose and L-arabinose.

A number of heavy metal salts, reducing agents, and iodoacetic acid were tested for their effect on hemicellulase and cellulase (Table III). On pectin polygalacturonase only the effect of silver nitrate, sodium sulphide, cysteine, and iodoacetic acid could be studied, because of pectate formation under influence of bivalent ions.

TABLE III

INHIBITION OF THE CELL-WALL-SPLITTING ENZYMES BY HEAVY METAL SALTS, REDUCING AGENTS, AND THIOL REAGENTS

Inhibitor*	Concentration	Mean enzyme activity (%)		
		"C_x" enzyme pH 5.5	Hemicellulase pH 5.5	Polygalacturonase pH 4.5
$AgNO_3$	1.29×10^{-2} *M*	0.0	13.8	75.0
$CuSO_4$	1.37×10^{-3} *M*	—	34.5	—
$ZnSO_4$	1.46×10^{-2} *M*	52.8	—	—
$Pb(NO_3)_2$	2.11×10^{-3} *M*	72.0	83.3	—
$Hg(Ac)_2$	1.37×10^{-3} *M*	62.1	41.2	—
$Hg(Ac)_2$ + cysteine (interaction)†	10^{-3} *M*; 10^{-3} *M*	65.1	40.4	—
Na_2S	10^{-2} *M*	113.6	118.2	89.3
Cysteine	10^{-2} *M*	100.0	91.9	75.0
Iodoacetic acid	10^{-3} *M*	36.3	94.6	64.3

*Inhibitor added to enzyme 5 minutes before experiment.
†Enzyme added to substrate after compound 1, followed 5 minutes later by compound 2.

Discussion

Hemicellulase and the "C_x" enzyme(s) are present as constitutive enzymes in ungerminated rust uredospores. During germination they increase remarkably although it should be mentioned that the total activity could not be recovered because of their extracellular character (37). Polygalacturonase proved to be adaptive and was the most active of the three enzymes in extracts of spores germinated on an agar pectin mixture. Dormant spores which failed to germinate well, also failed to produce more than a trace of polygalacturonase activity. Addition of 30 μg./ml. coumarin to the medium was effective in stimulating germination (38), but was unable to produce a corresponding stimulation of polygalacturonase production. The presence of coumarin and coumarin-like compounds in wheat is well known and there is a possibility that the rust resistance of certain varieties may be linked to their effect on germination and adaptive enzyme formation. Old spores contained more activity than fresh spores, although it is possible this difference is inherent in the spore collections themselves, rather than the aging process.

The hemicelluloses of resistant and non-resistant wheat strains were attacked with equal ease by the rust enzymes. This is in agreement with the fact that cell walls of resistant and non-resistant varieties are penetrated with equal facility.

Sodium carboxymethylcellulose was more readily attacked than barley β-glucosan by the spore extract. It is quite possible that the enzymes acting on both substrates are not the same; differences in structure of β-glucosan and CMC may partially, at least, account for this. The difference in degradation of the neutral mucilage of *Ceratonia siliqua* L. and the acid mucilage of linseed is even more striking. While the mannogalactan was readily split by the enzyme extract, the linseed mucilage was not. In this respect rust extract differs from extracts of other fungi like *Aspergillus niger* and *Aspergillus oryzae* where the enzymes are able to split acid and neutral mucilages with equal facility (37).

Rust enzymes were not very effective on *Tubera salep* mannan and barley hemicellulose. It is quite possible that the poorer solubility of these substrates is at least partly responsible.

Compared with the relatively fast decrease in viscosity of the CMC or mannogalactan the liberation of reducing sugars by the spore extracts was rather slow. This indicates that the internal linkages of the substrate molecules are attacked.

Another indication that the hemicelluloses are randomly split in smaller fragments by the rust enzymes is the production of di-, tri-, and higher polymers of xylose by the spore extract. However, the oligosaccharides could be formed by transglucosidation and thus may be artifacts of hydrolysis. Heavy metal salts diminish strongly the activity of the hydrolytic enzymes. The addition of cysteine hydrochloride did not reverse the inhibition caused by mercuriacetate. Sodium sulphide, however, activates the "C_x" complex and the hemicellulase activity while iodoacetic acid (SH reagent) proved to be inhibitory in all cases.

The complexity of the enzyme preparation makes it very difficult to draw conclusions concerning the number of hydrolases present and their properties. Purification and separation of the enzymes first must be undertaken to assess their specificity and properties. However, rust spores do possess active cellulases and hemicellulases and can be induced to produce a very active polygalacturonase.

Acknowledgment

The authors wish to thank Dr. T. Johnson of the Dominion Plant Pathology Laboratory of Winnipeg for a rust sample and the Hercules Powder Co. for the gift of carboxymethylcellulose samples. The assistance of Dr. F. J. Simpson in organizing and correcting the manuscript is gratefully acknowledged.

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THE STRUCTURE AND BEHAVIOR OF THE NUCLEI IN SPORES AND GROWING HYPHAE OF MUCORALES

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C. F. ROBINOW

Abstract

The behavior of the nuclei in resting and germinating spores and in growing hyphae of *Mucor hiemalis* has been followed during life with phase contrast microscopy. The nuclei consist of a dense central body, the nucleolus, surrounded by a shell of variable shape composed of optically uniform material of low density. The nuclei have been seen to divide by constriction. One half of the nucleolus and one half-shell of the low density material pass to each daughter nucleus.

All phases of nuclear division have been recognized in fixed and stained preparations. The nucleolus is readily stained by iron hematoxylin or gentian violet but is Feulgen-negative. The shell of low density has no marked affinity for hematoxylin and other basic or acid stains although it consists largely of granules and filaments which are Feulgen-positive. In these elements the chromosomes of the nuclei must somehow be contained but the size, shape, and behavior of individual chromosomes cannot yet be described. Division of the mass of chromatinic elements is direct and involves neither spindle nor metaphase plate. It is tentatively proposed that the chromosomes in the resting nucleus are already divided and segregated to opposite sides and that the constriction of the nucleus is merely the consummation of a kind of endomitosis initiated during the terminal stages of the previous division. Supporting observations have been made on *Mucor fragilis*, several other Mucorales, and two species of *Saprolegnia*. Uncertainties and controversies of the past can be explained in the light of the new findings.

Introduction

In the course of sexual reproduction the nuclei of many fungi give rise to chromosomes of more or less ordinary appearance and behavior. The literature on this phase of fungal life is extensive and well documented. By contrast there are few adequately illustrated descriptions of the structure and mode of division of the nuclei in *vegetative* parts of fungi. The present paper is an attempt to provide such a description of the vegetative nuclei of several species of *Mucor*. A further paper will deal with *Phycomyces*.

There are persistent indications, both direct and indirect, that vegetative nuclei of Mucorales (e.g. *Mucor*, *Rhizopus*, *Phycomyces*, *Pilobolus*) and also of *Saprolegnia* and *Empusa* divide in some unfamiliar way. A brief discussion of some of this evidence[2] seems worth while because, as further reports from this laboratory will show, some of the unusual features of mucorine nuclei are shared by the vegetative nuclei of members of other genera of the fungi.

It is generally agreed that the typical vegetative nucleus of the Mucorales takes the form of an easily stained dense body floating in a round or oval or spindle shaped space which some have described as clear and nonstaining and

[1]Manuscript received March 5, 1957.
Contribution from the Department of Bacteriology and Immunology, University of Western Ontario, London, Canada. Supported by a grant from the National Research Council of Canada.

[2]For a fuller bibliography of the older literature on the cytology of the Mucorales, see Cutter (8).

others as containing fine granules or threads. Previous accounts of the manner of division of vegetative nuclei, based mainly on fixed preparations, can be discussed under three headings:

1. How Do the Nuclei Divide?

There are many inconclusive descriptions of the nuclei in growing hyphae of Mucorales and related forms which reveal, directly or by implication, that their authors had failed to discover clear instances of familiar forms of division in their preparations. A good example of this is the detailed account of spore formation in *Pilobolus* by Harper (17). In this paper Harper had set himself the task of disproving the view of Léger (22) that the nuclei of Mucorales divide by constriction. However, after the study of many preparations Harper evidently preferred not to commit himself and, unable to account for periodic predictable increases in the number of nuclei during certain phases of development of the sporophore, ingeniously explained them by "the rapid multiplication of the nuclei by division". As Baird (2) has already pointed out, Harper neither states nor illustrates what mode of nuclear division his preparations suggested to him. In a well known paper on *Sporodinia grandis*, illustrated with unusually realistic drawings of vegetative nuclei (25 of them at high magnification), Keene (20) states that she never encountered nuclear divisions despite "a careful study of the nuclei in the germinating spores where divisions must occur". Five years later the same author (21), writing this time of *Phycomyces nitens*, confines herself to observe (of germinating spores): "They enlarge somewhat and one or two germ tubes push out. These grow for some distance during which time the number of nuclei present increases." One may conclude from this that nuclear division had then still not been observed by the writer. Equally uninformative are the many hundreds of neatly drawn nuclei, all of them seemingly at rest, in the numerous illustrations in the works of Ling-Young (24). Then again Schweizer (33), a resourceful and painstaking worker, confessed in 1945 that in the course of many years devoted to the study of *Empusa*, he had not once come across a dividing nucleus. (" . . . nicht ein einziges Kernteilungsstadium gesehen . . ."). What makes this statement so poignant is the fact that Schweizer is also the inventor of a special hematoxylin stain and has been commended by Cutter (9) on the heavy staining of the nuclei in his photomicrographs.

2. The Nuclei Divide Directly (Fig. 1*a*)

A failure to find dividing nuclei in central portions of a large mycelium or in sections of a growing fruiting body may be due to the scarcity of divisions in the one and their marked periodicity in the other. But neither of these explanations can account for the repeated failure of skilled observers to see divisions in germinating spores and growing hyphae of young mycelia which are growing continuously and are crammed with nuclei. One is forced to conclude that nuclear divisions in these structures are disguised in such a way that they are not seen by those who only look for familiar appearances.

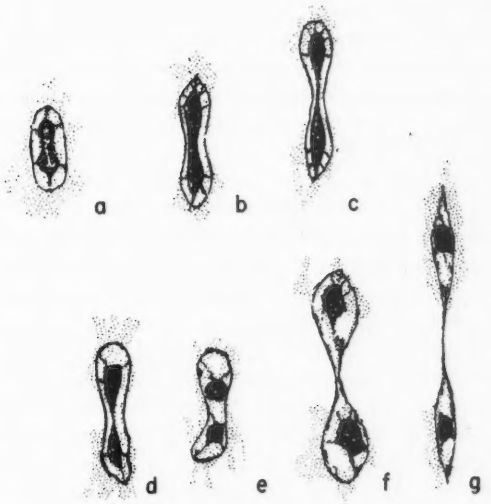


FIG. 1a

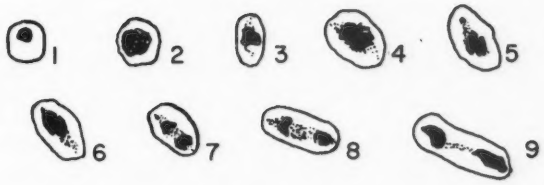


FIG. 1b

FIG. 1*a*. Nuclei of *Saprolegnia* in various stages of direct division. After Smith, 1923.
FIG. 1*b*. Mitosis in *Rhizopus sexualis* according to Callen, 1940; (1) resting stage, (2–3) prophase, (4–6) metaphase, (7–8) anaphase, (9) telophase.

This suspicion is strengthened by the peculiar character of the few and very similar drawings of dividing phycomycete nuclei in the old as well as in the more recent literature. To judge by these pictures, division of the nucleus is achieved by the constriction of the central body and of the clear zone around it and is not accompanied by the emergence of chromosomes. In other words: the nucleus has essentially the same structure during and between divisions. There is the same deeply stained central mass—now single, now constricted—surrounded by the same nonstaining or finely granular halo. This form of division has been found in many Mucorales by Léger (22) and in *Saprolegnia* by Trow (38) and again by Smith (36) (see Fig. 1*a*). Also in the course of a description of the behavior of the nuclei in *Saprolegnia Thureti* Dangeard (10) admitted as recently as 1930 that: "Le fait d'avoir examine des milliers et

des milliers de noyaux nous rend assez perplex jusqu'ici sur l'existence de véritables mitoses dans cette espèce, en ce qui concerne le mycelium; partout nous n'avons constaté qu'une fragmentation du nucléole, lors de la division du noyau qui se présente alors comme une division directe".[3]

3. The Nuclei Divide by Mitosis

There are, lastly, those who see the central basophile body of the dividing nucleus as a mass of chromosomes and interpret the process of division as a form of mitosis (4, 8, 27), Fig. 1*b*. This sounds more plausible than the amitosis described in the previous section but when representative illustrations are compared, e.g. Figs. 1*a* and 1*b*, then it is seen that they both reflect the same kind of observations and that in neither is there good evidence of chromosomes. The fact that some division figures suggest very strongly a direct mode of division has not been overlooked by the advocates of mitosis but it was held that amitosis is restricted to "the older vegetative mycelium" or "those portions of the thallus where active cytoplasmic movement has ceased". However, the distinction between the two modes of division, as described by Cutter (8, pp. 484–485) is purely verbal and does not suggest any real difference in the behavior of the central "chromatic mass" in the two instances.

It is no longer profitable to discuss the relative merits of different views of the mode of division of vegetative nuclei of Mucorales and of *Saprolegnia* because the assumption, common to them all, that the chromatin of the dividing nucleus is inside, or derived from, the central body has become highly improbable through the demonstration by Sjoewall (35), confirmed in the present study, that the central body of *Mucor* nuclei is Feulgen-negative and that even during division Feulgen-positive matter is only found in the clear, non-basophile area surrounding it. Using the hydrochloric-acid – Giemsa method, Robinow (31, 32) had independently come to the same

[3]It might be thought that the findings of Smith and Dangeard have been rendered obsolete by the work of Shanor (34) and Ziegler (41), who have demonstrated chromosomes and spindles, mitosis, and meiosis in *Saprolegnia*. Strictly speaking that is not so. The two authors have not described divisions of nuclei in growing vegetative hyphae. They have described nuclear divisions in sexual organs of *Saprolegnia*. This difference is probably significant. It is certainly puzzling, because Shanor's and Ziegler's method of staining—gentian violet, iodine, alcohol—does not reveal the Feulgen-positive material of nuclei in growing hyphae of *Saprolegnia* and when their chromatin is rendered visible with the Feulgen reaction it is seen that the division of these nuclei does not involve metaphase plates (unpublished findings by A. Bakerspigel). One is forced to the conclusion that the chromosomes in the sexual organs behave rather differently from those in growing hyphae. However, the example of the spurious "mitosis" in *Rhizopus* (4) makes it desirable that the work of Ziegler be repeated with the Feulgen technique.

More pertinent to the present work is the report by Hatch (18) of ordinary mitosis in the tips of growing hyphae of *Allomyces arbuscula*. Hatch used the Feulgen reaction but his evidence is incomplete. Prophase and the reconstruction of daughter nuclei are not shown and the nuclei lack the central nucleolus which they are said to possess during life. The fate of the nucleolus during division is not discussed. Hatch fixed his material with mercuric chloride, a reagent which the experience of this laboratory has shown to give misleading images of the structure of fungal nuclei. Further distortion is likely to have occurred during dehydration and paraffin embedding. At present the fragmentary work of Hatch contrasts abruptly with many other observations both old and new which are in far better agreement with the behavior of living nuclei in the hyphae of higher phycomycetes.

conclusion regarding the distribution of chromatin in the vegetative nuclei of several fungi, including *Mucor hiemalis*. Although his results with resting *spores* of *Mucor* were quite definite, Sjoewall (35) did not obtain clear pictures of dividing nuclei in *growing hyphae*. Fresh observations on the mode of division of vegetative nuclei of Mucorales are clearly needed and are offered in the present paper.

Materials

Most of the observations to be described were made on vegetative spores and young mycelia of *Mucor hiemalis* and *M. fragilis*. Two strains of the former ("plus" and "minus") were obtained from the Botany School, Cambridge University, through the courtesy of Dr. John Rishbeth. Most of the work was done with the minus strain. *M. fragilis* was isolated from soil. Identification of this species was kindly undertaken by Dr. W. Hesseltine of the U.S. Agricultural Research Service at Peoria, Illinois.

The fungi were grown in the room at temperatures around 22° C. Stock cultures were stored at 4°–6° C. The medium used throughout was composed of yeast extract (Difco) 0.5%, glucose 2%, and agar 1.5%.

Additional observations were made on the nuclei of *Mucor recurvus* and of *Endogone sphagnophila* in preparations made by Mr. A. Bakerspigel. *Phycomyces blakesleeanus*, of which several strains have been examined will be dealt with in a separate paper. Of *Saprolegnia* enough has been seen in slide cultures and variously stained preparations (also prepared by Mr. A. Bakerspigel) to permit comparison with preparations of *Mucor*.

It should be understood at the outset that the literature on the cytology of the Mucorales indicates that they all have the same kind of nuclei and that this similarity probably extends to other genera of the phycomycetes as well. References to vegetative nuclei of *Saprolegnia* or *Empusa* are therefore not unreasonable, though at first they may seem out of place in a paper primarily devoted to two species of *Mucor*.

Methods

A. Phase Contrast Microscopy of Living Spores and Growing Hyphae

1. Spores

Resting spores (conidia) were examined in 25% non-nutrient gelatin to depress their awkwardly high natural refractility. This method was adopted after the work of Mason and Powelson (25) had shown that media with much gelatin increase the clarity of internal detail in living bacteria.

2. Growing Hyphae

To prepare cultures of growing hyphae, object slides not thicker than 1 mm. were dipped into freshly melted yeast extract – glucose agar and placed upright until all but a thin layer of agar had drained away. After the agar had set, the process was repeated with the ends of the slide reversed to prevent

the agar film from becoming wedge-shaped. After the second coat of agar had set, all of it except a narrow oblong measuring about 3 by 8 mm. was scraped off the slide with the aid of the flattened tip of a length of resistance wire.

The agar surface was sparingly streaked with spores using fine flexible glass fibers obtained by drawing out slender glass rods or capillary tubing. A No. 1 oblong cover slip was then gently lowered from one side on the inoculated agar and sealed around the edges with wax. Germ tubes suitable for observations of living nuclei were usually obtained after 6–7 hours. The microscope (Bausch and Lomb, dark contrast) was illuminated with the light of a tungsten ribbon lamp passing through a Baird Associates interference filter with maximum transmission at 5460 Å. Photographs were taken on Kodak Royal Pan cut film at a magnification of 1333 times and printed at a twofold enlargement.

B. Preparation of Materials for Examination after Fixation and Staining

Most observations were made on preparations obtained by either the one or the other of two procedures differing in the manner of handling the fungus as well as in the choice of fixative and stain.

Method I. Agar Film–Osmium (or Sanfelice) – Hematoxylin Preparations

Thin (single-dip) films of yeast extract – glucose agar on No. 1 cover slips 22 mm. square were inoculated with spores with the aid of a fine glass fiber and incubated in a moist atmosphere. Several rows of spores were planted for preparations of germ tubes. A small, single, and centrally placed inoculum and a weaker nutrient medium was used when large mycelia were desired.

At the appropriate stage of growth, cultures were exposed for 5 minutes to osmium tetroxide vapor. Fixed cultures were stored in 70% alcohol. It would have been most satisfactory if osmium tetroxide could have been used for all preparations, because among several that were tried this reagent achieved equally good preservation of nuclei, mitochondria, and the texture of the cytoplasm. Unfortunately most stains gave poor results after osmium fixation and even with iron alum hematoxylin, it proved difficult to differentiate nuclei and mitochondria from the intensely basophile cytoplasm of the germ tubes. Satisfactory preparations were obtained of only the broad hyphae near the growing edge of large mycelia. This was disappointing because it is in small mycelia or in germ tubes that one finds the highest concentration of dividing nuclei. Fixation with Sanfelice, adopted on the advice of Prof. C. A. Callan of St. Andrew's University, provided a compromise solution. This fixative coarsens the structure of the cytoplasm and destroys the mitochondria but it preserves the nuclei well and even in germ tubes allows them to be clearly differentiated from the cytoplasm.

Osmium- or Sanfelice-fixed cultures were mordanted for 7–15 hours in 2% iron alum and stained with the half oxidized hematoxylin of Baker and

Jordan (3). They were differentiated under the microscope in 2% iron alum or in a saturated solution of picric acid, washed with tap water, dehydrated for a few minutes each in 70%, 95%, and 100% alcohol, carried through xylene, and mounted in "DPX" synthetic resin.

Hematoxylin, as will presently be apparent, is not a useful stain for the demonstration of chromatin in vegetative nuclei of phycomycetes. It is therefore necessary to make use of the Feulgen procedure for the detection of sites of desoxyribonucleic acid (DNA). This method involves treatment with *N*/1 HCl at 60° C., a procedure which unfortunately precludes the use of the thin agar films of Method I. Moreover, the Feulgen reaction is more distinct and the affinity of hydrolyzed nuclei for stains in general is greater after fixation with *acetic acid alcohol* than it is after fixation with osmium tetroxide. For these reasons the following procedures were adopted for most of the preparations intended to show the distribution of chromatin (i.e. the stainable matter of the chromosomes) in the nuclei.

Method II. 1. Handling, Fixation, and Staining of Materials in Which It Was Intended to Stain the Chromatin of the Nuclei

(*a*) *Spores*

Bunches of sporangiophores, plucked from the culture dish by twisting them around a wire loop bent into the shape of a shepherd's crook, were rubbed over grease-free No. 1 cover slips. The thin films of spores so produced dried within a few seconds and were at once immersed in either acetic acid alcohol (1:3) or the fixative of Newcomer (28) where they remained for 10 minutes. If fixed films were not used immediately they were either stored in 70% alcohol or allowed to remain in Newcomer's fixative at 4° C.

(*b*) *Germinating Spores and Young Mycelia*

(i) *Cultures on cellophane.*—These were grown on the surface of very thin cellophane (dialysis grade), lying on yeast extract – glucose agar in a Petri dish. The advantages of this method for a variety of purposes were first seen by Fleming and Smith (12) and have been emphasized afresh by Carmichael (5). Germ tubes and young mycelia were fixed for 10 minutes in acetic alcohol, occasionally also in Newcomer's fixative, and were stored in 70% alcohol. Much of the growth usually came adrift in the fixative and all attempts to prevent this have proved unavailing.

(ii)—A small number of preparations was made by pressing young growth on agar against thin wet films of fresh undiluted egg white on cover slips and then pulling the cover slip away with a quick, abrupt movement. The wet films were then fixed in acetic acid alcohol.

Method II. 2. Staining

(*a*) *Acetocarmine*

In the preparation and use of this reagent I have followed the directions of Geitler (13).

The combined stain–fixative was prepared by dissolving a gram of carmine in 100 ml. of 45% acetic acid and allowing the solution to simmer for 1 hour. Small pieces of fungus-bearing cellophane were either placed directly in a drop of the reagent and covered with a No. 1 glass slip or were first fixed for a few minutes in acetic acid alcohol.

(*b*) *Feulgen Technique*

Pieces of cellophane bearing fixed fungal growth were transferred directly from 70% alcohol to *N*/1 HCl at 60° C., treated for 9 minutes, rinsed with water, left in Schiff reagent in well sealed "Columbia" staining jars for 2–4 hours, rinsed rapidly with ten 10 ml. lots of sulphur dioxide water, were then washed for 20 minutes in several changes of tap water, and following the example of McIntosh (26) were mounted, growth upwards, in a drop of acetocarmine, covered with a No. 1 glass slip, and sealed with wax. In every instance the outcome of the reaction was first observed in preparations mounted in water. Unhydrolyzed controls were also examined.

To prevent the hyphae from detaching themselves from the cellophane during hydrolysis a coat of collodion was sometimes applied before the preparation was exposed to the acid. This was done by carrying small pieces of fungus-bearing cellophane rapidly from 70% alcohol through 95% and 100% alcohol into a 0.5% solution of "Parlodion" in equal parts of absolute alcohol and ether. After a few seconds in this solution the cellophane squares were taken out and held in air until they appeared covered by a thin, gray, just tacky film of collodion. They were then returned to 70% alcohol. The presence of the collodion did not seriously interfere with the examination of stained specimens but sometimes it was removed before staining by carrying the now hydrolyzed specimen once more through alcohols into alcohol–ether (without collodion) and back again into water.

The Schiff reagent was prepared in the ordinary way: 200 ml. of boiling distilled water were poured over 1 g. of "Diamant Fuchsin" (sold by Chroma Gesellschaft, Schmid and Co., Stuttgart, Germany, and distributed by Roboz Surgical Instrument Co., Washington, D.C.). To the cooled and filtered solution were added 1 g. of potassium metabisulphite and 20 ml. of *N*/1 HCl. The reagent bleaches overnight to the traditional pale straw color, is perfectly clear, and requires no filtering through charcoal. It has given us a somewhat brighter Feulgen reaction than other brands. In the course of this work, which has included studies of fungi not described in the present paper, it was noted that there are great differences in the maximum intensity of the Feulgen reaction of the nuclei of different fungi after fixation with acetic acid alcohol. The red color of the positive reaction is delicate but distinct in *Mucor*, very faint in *Geotrichum candidum*, and very strong in conidia of *Empusa*. A correlation probably exists between the startling brightness of the Feulgen reaction in *Empusa* and the unusual affinity of the nuclei of this fungus for hematoxylin, evident in the work of Schweizer (33).

(*c*) *Hydrochloric Acid–Giemsa Technique*

Specimens fixed and hydrolyzed as under *b* were stained for several hours or overnight in Gurr's Giemsa R 66 (two drops per ml. of Gurr's "Giemsa buffer" at pH 6.3 or slightly higher). If necessary they were differentiated under a water immersion lens in a Petri dish of distilled water to which one to three wire loopfuls (3 mm. across) of glacial acetic acid had been added. Finished preparations were mounted in the staining solution or in buffer, covered with a No. 1 glass slip, and sealed with wax.

The Feulgen reaction as well as hydrochloric acid–Giemsa staining have been used both after osmium fixation and after acetic acid – alcohol fixation. The chromatin had the same distribution in the nuclei of all four kinds of preparations but after acetic acid alcohol there was much better contrast between the nuclei and the cytoplasm. The pictures of living nuclei and Fig. 2 provide a common point of reference for the results obtained with the two fixatives as well as for the Sanfelice mixture, which was used only in conjunction with iron hematoxylin. *Feulgen acetocarmine or hydrochloric acid – Giemsa after acetic acid alcohol fixation have proved to be the methods of choice for the staining of vegetative nuclei.* In the present study iron hematoxylin has been used chiefly to reveal the behavior of the nucleolus during division and to provide a link with the illustrations in the older literature.

Photography

Photomicrographs were usually taken of wet mounts but hematoxylin preparations were dehydrated and mounted in balsam before they were photographed. The lamp and filter were those described under A2, set up to provide Koehler illumination. The microscope was fitted with a Zeiss achromatic–aplanatic substage condenser (N.A. 1.4) used with oil between top lens and slide, a Zeiss apochromatic objective lens ×90 (N.A. 1.3), and a ×15 Zeiss compensating eyepiece. Photographs were taken at an initial magnification of 1800 times and were enlarged twice in printing.

Results

1. Nuclear Division as Observed in Living Hyphae (Figs. 2 and 4–6)

In living hyphae phase contrast microscopy reveals four kinds of structures: minute dense *granules* carried swiftly towards the tip and back again by invisible currents; *mitochondria*, short and in vivid Brownian motion at the tip of growing hyphae, but long and filamentous and relatively slow moving in more proximal parts; brightly shining sap *vacuoles*, continuously changing shape and position; and, lastly, the *nuclei*. Only the latter will be discussed.

The shape of the nuclei varies continuously. Rounded, oval, or teardrop shapes are most common but the nuclei may also be angular or drawn out into narrow tails. The flame-like variability of mucorine nuclei is reflected in Keene's drawings of stained germ tubes of *Sporodinia grandis* (20) and has also been noted in living preparations of several other fungi by Dowding and Bakerspigel (11) and Girbardt (14). The outer region of the nuclei is of an

even, unchanging, low density. Its contours are smooth but there is no indication of a limiting membrane. The interior of the nucleus is occupied by a variously shaped dense central body which will be referred to as the nucleolus.

Most favorable for the study of the nuclei were germ tubes in which the length of the developing hypha had reached about three times the diameter of the bulbous proximal part that originally was the spore and in which the highly motile, optically troublesome vacuoles had not yet developed. Nuclei about to enter division were recognized by their size and the relatively long distance separating them from their fellows. It was soon seen that small nuclei, close to each other, are products of a recent division, and not likely to divide again during the next hour or so. The main features of the process of nuclear division observable during life are drawn in Fig. 2, which is based on a

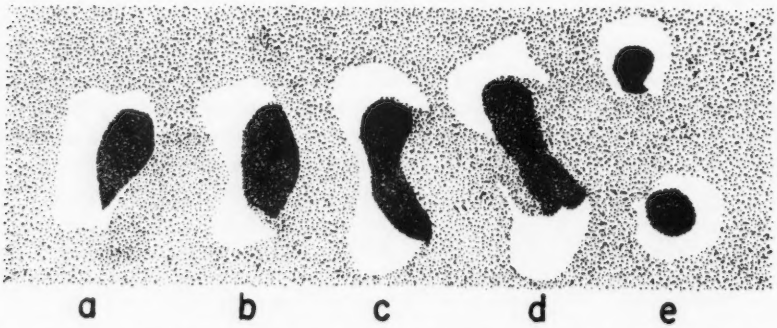
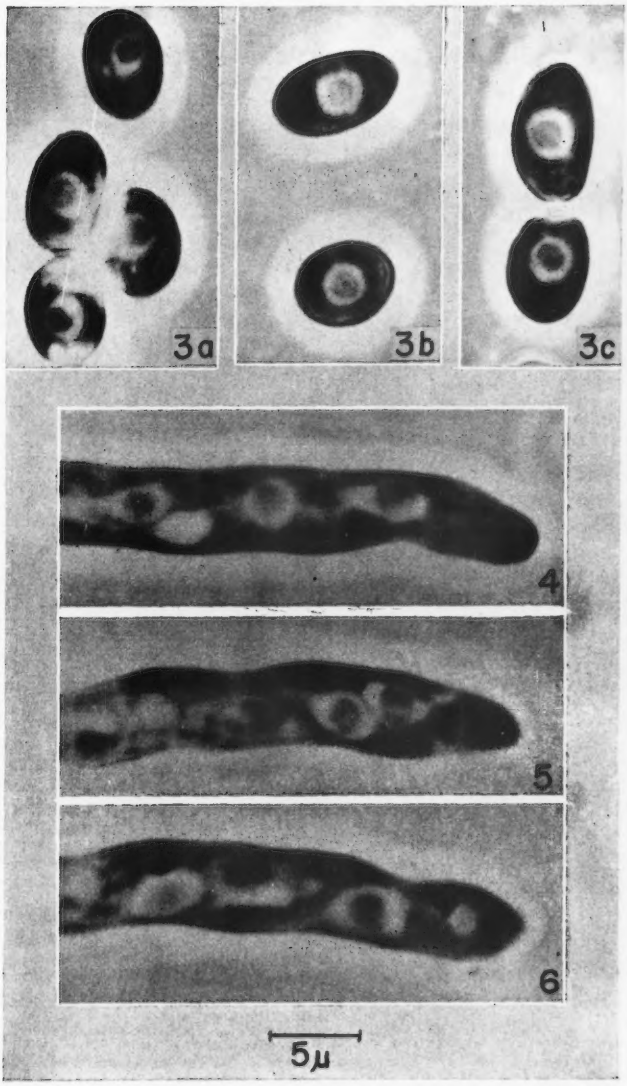


FIG. 2. Successive stages of the division of a living nucleus of *M. hiemalis* as seen by phase contrast microscopy. The interval between *a* and *e* is 6 minutes. The drawing is based on freehand sketches made by the side of the microscope. Observation of June 26, 1956.

series of rapid sketches made by the side of the microscope. These drawings lack depth but are accurate in the main features. I have watched many nuclei divide and several divisions have been followed by other members of the laboratory. Division is accomplished in 2–4 minutes. Separating nuclei twist and turn and so far I have not been able to record their movement in photographs, although in another fungus, *Geotrichum candidum*, time-lapse photographs were obtained without difficulty. A detailed description of the division maneuvers will not be presented until it can be supported by photographs. Meanwhile three constant features of the division process may be pointed out: the straightening of the contours on one side of the nucleus at the beginning of division, the lack of visible changes of texture in the transparent part of the nucleus, and the elongation, constriction, and division of the nucleolus. Daughter nuclei are, at first, relatively small and, in optical section, do not add up to the area occupied by the nucleus at the beginning of its division. The extent of these changes and the growth of the nuclei between divisions need to be measured.



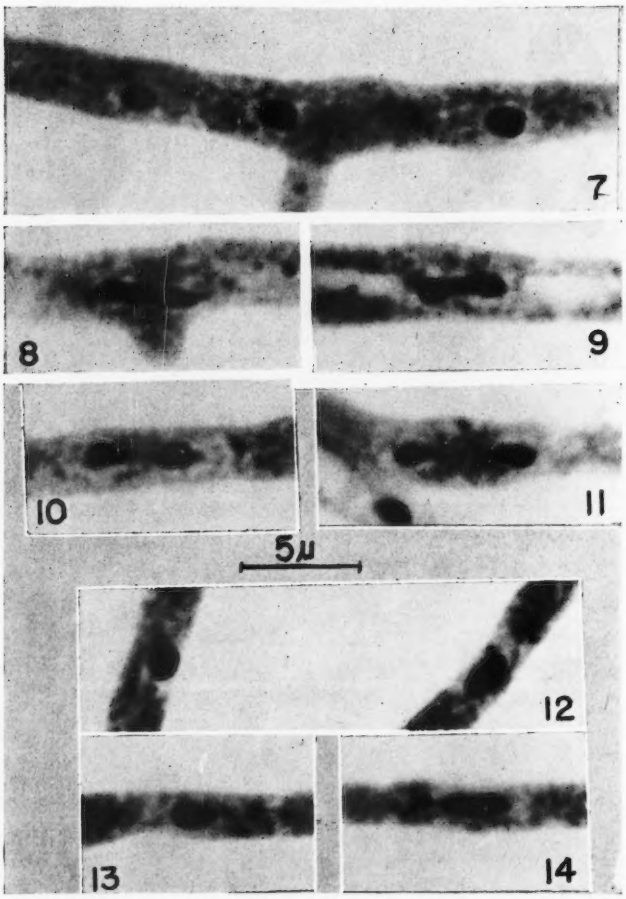
All figures on Plates I–VII are of *Mucor hiemalis*.

FIGS. *3a*, *3b*, *3c*. Living spores mounted in 25% non-nutrient gelatin. Phase contrast microscopy. Compare with stained preparations on Plate VII.

FIGS. 4–6. Tips of growing hyphae (germ tubes). Phase contrast microscopy. Note the irregular shapes of the nuclei, the large dense nucleoli, and (in FIG. 4) the filamentous mitochondria.

The magnification of all the figures is 2670 times and is indicated by the scale underneath FIG. 6.

PLATE II



All figures are from dehydrated permanent preparations fixed with Sanfelice and stained with iron hematoxylin. The magnification of the figures on this and all remaining plates is 3600 times.

FIGS. 7–11. Examples of frequently seen phases of nuclear division. From 7-hour mycelia growing in thin agar films on cover slips. Comparison with the nuclei of living hyphae (FIGS. 4–6) reveals slight shrinkage. Note the deeply stained nucleoli surrounded by unstained halos which, here and there, are traversed by delicate threads. The nucleolus divides by constriction and its halves become part of the daughter nuclei. Compare with Feulgen preparations on Plates IV and V.

FIGS. 12–14. Examples of nuclei with associated granules of unknown significance. In the past, nuclei having the configuration of the one on the left in FIG. 12 may sometimes have been mistaken for a packet of chromosomes between centrioles.

2. Observations on Fixed Preparations

(*a*) *The Nuclei in Hematoxylin Preparations* (*Method I*)

The nuclei in Sanfelice–hematoxylin preparations of germ tubes and young mycelia (Figs. 7–11) resemble living nuclei in cultures of comparable age examined by dark phase contrast microscopy. They have a massive nucleolus which holds hematoxylin far more tenaciously than the narrow band (or shell) of transparent material surrounding it. I have not been able to detect the presence of a distinct and continuous limiting membrane at the surface of the nuclei. Here and there delicate threads may be seen stretched out between the nucleolus and the ill-defined margin of the nucleus. These strands probably correspond to the fine reticulum which earlier observers have described in mucorine nuclei. A survey of the illustrations in the literature on the Mucorales shows that the nuclei take on the same appearance—conventionally a solid nucleolus within an unstained oval—regardless of whether they have been stained by iron alum hematoxylin or by simpler, direct stains. In my own work I have found this to be true of Ehrlich's hematoxylin, basic fuchsin, acid fuchsin, methylene blue, and Newton's gentian violet – iodine, the latter used as recommended by Baker and Jordan (3). Aceto-carmine, which instantly gives useful information on the state of the nuclei in plant and animal tissues and in protozoa, does not immediately give a clear picture of the nuclei of Mucorales and was only used as an adjunct to the Feulgen procedure.

Osmium-fixed nuclei are more homogeneous and have smoother contours than those in Sanfelice preparations but share with them the lack of a visible limiting membrane. The nuclei illustrated in Plate III, although quite representative of *Mucor*, are not directly comparable with those of Plate II. This is not only because they are fixed differently but because they come from the broad hyphae of 18-hour-old mycelia which contain nuclei of a much wider range of shapes and sizes than the germ tubes and early mycelia which supplied the nuclei of the hyphae in the photographs on Plates I and II. With their indistinct margins and angular contours the osmium-fixed nuclei in Figs. 15–18 closely resemble those which Keene drew and described in Flemming-fixed *Sporodinia grandis* (20, Plate XXV, Fig. 2). It will be noted that the substance of the osmium-fixed nuclei is more deeply stained than that of the Sanfelice-fixed nuclei of Plate II. However, it would be rash to assume, because of this, that it is the chromatin of the nuclei that has retained the hematoxylin. It will be seen that the nuclei have only retained as much of the stain as the mitochondria, structures whose affinity for hematoxylin has been traced to their contents of lipids and ribonucleic acid (40).

Fixed preparations of germ tubes and young mycelia abound in configurations which experiences with living hyphae allow one to identify as stages in the division of the nuclei. A few representative examples are shown in Figs. 8–11 and Figs. 17 and 18. The straightening of nucleus and nucleolus early in division (Figs. 7 and 8) and the constriction and partitioning of the latter are clearly shown. The aura around the nucleolus, unstained except where it

is traversed by delicate threads, also constricts and divides and, most remarkably, it does so without changing its appearance or acquiring temporarily a greater affinity for hematoxylin. Division figures of this kind are not only numerous, they are the only ones to be seen in fixed preparations and closely resemble the division figures of *Saprolegnia* nuclei seen by Smith (36) and copied in Fig. 1*a*. The process of division which they reflect is also the only form of division encountered during many long periods of continuous observation of living nuclei.

In the nuclei in Sanfelice preparations one or two small, round, deeply stained granules were often seen close to the nucleolus and, in some instances, connected with it by a fine stalk. In one sample 31 out of 110 nuclei had such a companion. Similar but less conspicuous granules can be recognized in osmium-fixed preparations but none were seen with certainty in preparations stained by the Feulgen or hydrochloric acid–Giemsa techniques. Possible explanations of the nature of these granules will be discussed at the end.

Under the conditions under which I have examined them, living *Mucor* nuclei did not contain, and at no time during their division gave rise to, visible chromosomes though it is conceivable that chromosomes would have been revealed by a different form of phase contrast or by the use of ultraviolet light. Hematoxylin has proved equally unhelpful although it has given some indication that the material surrounding the nucleolus is not entirely homogeneous. The nucleolus being ruled out as the site of the chromosomes on account of its negative Feulgen reaction, these evasive entities had to be searched for in the non-basophile part of the nucleus.

(*b*) *The Nuclei in Feulgen and in Hydrochloric Acid–Giemsa Preparations* (Plates IV and V)

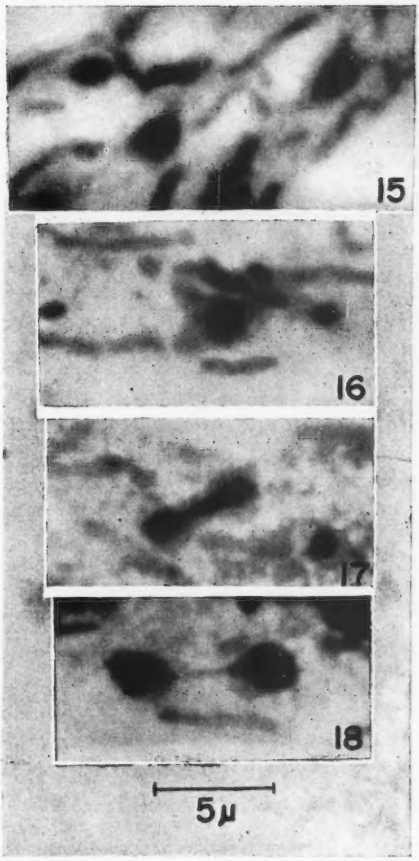
(*i*) *The Nuclei of Growing Hyphae*

The material with no marked affinity for ordinary stains which surrounds the nucleolus is composed of many small particles that give a positive Feulgen reaction. In some nuclei the particles form a tightly woven net (Fig. 22); in others they seem to be closely packed in no particular order. The nucleolus itself is Feulgen-negative. These findings are in agreement with the observations of Sjoewall (35) and recall those of Heim (19) on vegetative nuclei of *Plasmodiophora*.

Comparison of the pictures of living nuclei on Plate I with those of Feulgen preparations on Plates IV and V reveals that acetic acid alcohol preserves very well the varied, angular shapes of the nuclei and the way in which their corners tend to be drawn out into slender points or trailing filaments. The non-dividing nuclei in Figs. 20, 21, 22, 23, and 28 are good examples of this.

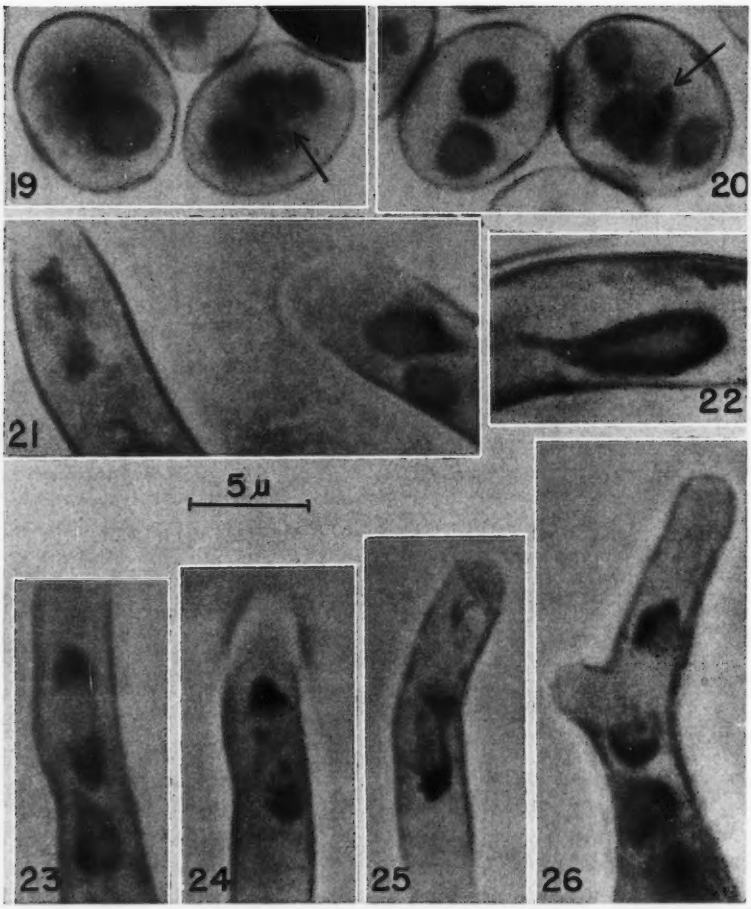
In the description of living nuclei earlier in this paper, it was pointed out that the transparent part of the nucleus suffers no obvious changes in texture or density during the process of division. In Feulgen preparations this is reflected in the remarkably smooth transition between the shapes of resting

PLATE III



All figures from preparations that had been fixed in osmium tetroxide vapor, stained with iron alum hematoxylin, dehydrated, and mounted in "DPX", artificial resin.

FIGS. 15–18. Nuclei and mitochondria in hyphae of extensive 18-hour mycelia growing in thin agar films on cover slips. Note variable shape and size of the nuclei and stages in their direct division.



All figures on this plate are from preparations on cellophane, fixed with acetic acid alcohol, stained by the Feulgen procedure, and thereafter mounted in acetocarmine.

FIGS. 19 and 20. Swollen spores ready to germinate after 3 hours on yeast extract glucose agar. Arrow in FIG. 19 points to a dividing nucleus. Note large nucleus in FIG. 20 from whose periphery strands of chromatin extend into the cytoplasm.

FIGS. 21–26. From 7-hour mycelia.

FIG. 21. At the left a nucleus with granular chromatin, half way through division. At the right two non-dividing nuclei.

FIG. 22. Relatively large nucleus with net-like shell of chromatin.

FIGS. 23–25. Terminal stages of nuclear division.

FIG. 26. Two nuclei, products of a recent division, with dense crescents of chromatin.

and dividing nuclei, which, at any rate in the early phases, involves only a change of form and not of structure (compare for example the nearly divided nucleus at the left in Fig. 19 with the nondividing nuclei in Figs. 20, 21). It is only when the chromatin of the nucleus has broken apart into two half-shells or cups—crescents in optical section—and after these have suffered some degree of contraction, that there is a noticeable increase in the strength of the Feulgen reaction (see Figs. 23–26 and the nuclei in Figs. 30, *c*; 31, *d*; and 32).

The granularity and frayed edges of the chromatic part of the nuclei in Feulgen preparations contrast with the even transparency and smooth contours of the corresponding region of the living nucleus. This difference, which is equally noticeable after fixation with osmium tetroxide, can be explained by assuming that during life the Feulgen-positive granules are embedded in some material of the same optical density as themselves but of different composition. Absorption of the less specific Giemsa stain by both the granules and the matrix would explain the greater density of the nuclei in hydrochloric acid – Giemsa preparations. A matrix of some kind is in fact visible in the nuclei of the spores which will be described in the next paragraph.

3. The Nuclei in the Spores of *Mucor hiemalis* (Figs. 3*a*, 3*b*, 3*c*, 33, 34)

Most of the spores of the two strains of *M. hiemalis* examined had one nucleus; a few had two nuclei. Spores are so highly refractile that their interior is not easily made out unless the spores are mounted in a medium of suitably high refractive index. Twenty-five per cent non-nutrient gelatin, suggested by the work of Mason and Powelson (25), proved satisfactory for this purpose (Figs. 3*a*, 3*b*, 3*c*). The living nuclei are very similar to the nuclei in growing hyphae but are more nearly round.

Fixed spores contain loose arrangements of short Feulgen-positive threads and granules embedded in a matrix. In hydrochloric acid – Giemsa preparations the matrix seems to be of the same nature as the not always clearly differentiated nucleolus. The nuclei of resting spores are less chromatic and more loosely knit than the nuclei of germinating spores and growing hyphae and have very diffuse margins. It is these features which at first suggest that Fig. 34 must be out of focus but this is disproved by the reasonable sharpness of the detail in the nuclei of the two spores marked with arrows.

After a short while on a nutrient medium the spores lose most of their troublesome refractility and become suitable for phase contrast microscopy in ordinary agar media. The nuclei divide several times before the germ tubes are pushed out (Figs. 19, 20) and during this time, to quote Harper (17) on spores of *Pilobolus*, which seem to behave like those of *Mucor* in the present work: "The nuclei undergo considerable amoeboid changes of form and seem even to change position in the spore by this means". Movement seems reflected in the strands of chromatin which are trailing away from the edge of the large nucleus in Fig. 20.

4. *Mucor fragilis* (Figs. 35–40)

The nuclei of this delicately proportioned organism are smaller than those of *Mucor hiemalis* but are constructed in the same way. In resting spores the nuclei have again a scrambled, spongy structure and in swelling spores and germ tubes the nuclei apparently divide in the same direct way as those of the larger species.

Discussion

The observations described in the preceding pages have revealed something of the structure and mode of reproduction of *Mucor* nuclei and at the same time have raised some new questions. It will be convenient to deal with some points of structure first and leave the matter of division to the end.

1. The Absence of a Readily Visible Membrane at the Surface of the Nuclei

There seems to be a correlation between the shape of the nuclei and the presence of a visible membrane at their surface. Living nuclei of *Mucor* have fleeting, irregular shapes and soft contours. Likewise no nuclear membrane is found and nuclear margins are indistinct in those preparations where fixation has preserved the natural irregularities of form of the nuclei (see Keene (20) and the photographs on Plates II–VII of the present paper). Various damaging influences cause the nuclei to become oval or spherical and it is then that limiting membranes become distinctly visible. In slide cultures of *Mucor* and *Saprolegnia* this was repeatedly observed in the nuclei of hyphae that had ceased growing and whose cytoplasm had begun to disintegrate. Rapid transformation of the nuclei into spheres with dark sharply drawn margins was also seen on one occasion when some ethanol had penetrated into a slide culture. It is understandable then, that the most definite references to a nuclear membrane are to be found in those papers (8, 18, 22, 37) which picture the shape of the nuclei as perfectly (and most unnaturally) round or oval.

Our observations were made independently of those of Girbardt (14), who noted the "lobed", variable shapes of living nuclei of the basidiomycete *Polystictus* and whose remarks on the smoothing effects of fixatives are in accord with our own experience. Interesting is Girbardt's remark (14) that the change to the spherical shape is reversible.

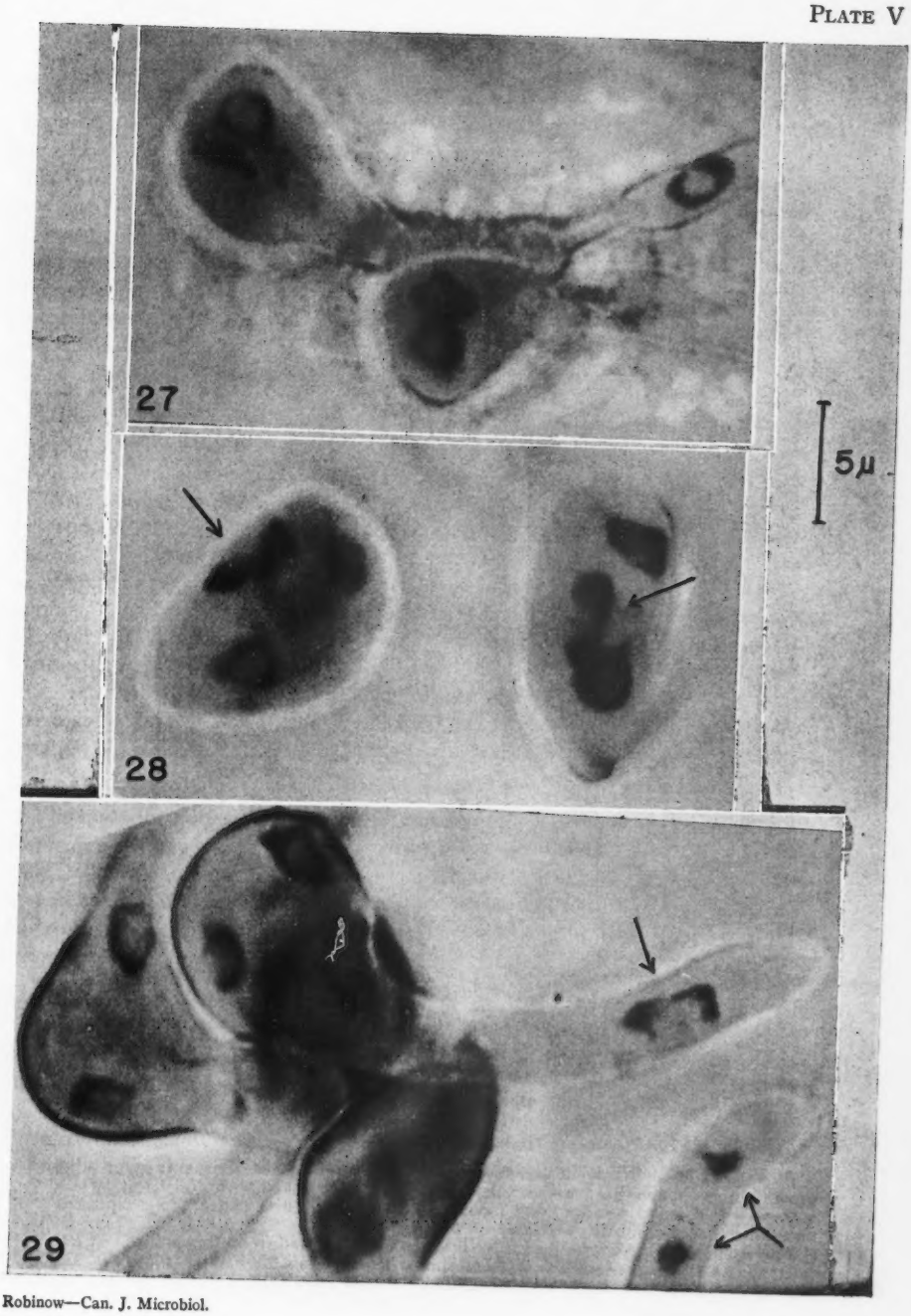
Definite envelopes around nuclei have recently been demonstrated in electron micrographs of thin sections of zoospores of *Allomyces* (39) and of hyphae of *Coccidioides immitis* (29). It remains to be seen what electron microscopy will show at the surface of the nuclei of *Mucor*.

All figures on this plate show spores germinating on cellophane, fixed with acetic acid alcohol, stained by the Feulgen procedure, and thereafter mounted in acetocarmine.

FIG. 27. Representative shapes of non-dividing nuclei, with their shells of tightly packed chromatin particles. Compare with photographs of living nuclei in FIGS. 4–6, Plate I.

FIGS. 28 and 29. Germ tubes containing both non-dividing and dividing nuclei. The latter are indicated by arrows. In the right bottom corner of FIG. 29 are two clusters of chromatin particles, products of a recent division, comparable to those in FIG. 24, Plate IV.

PLATE V



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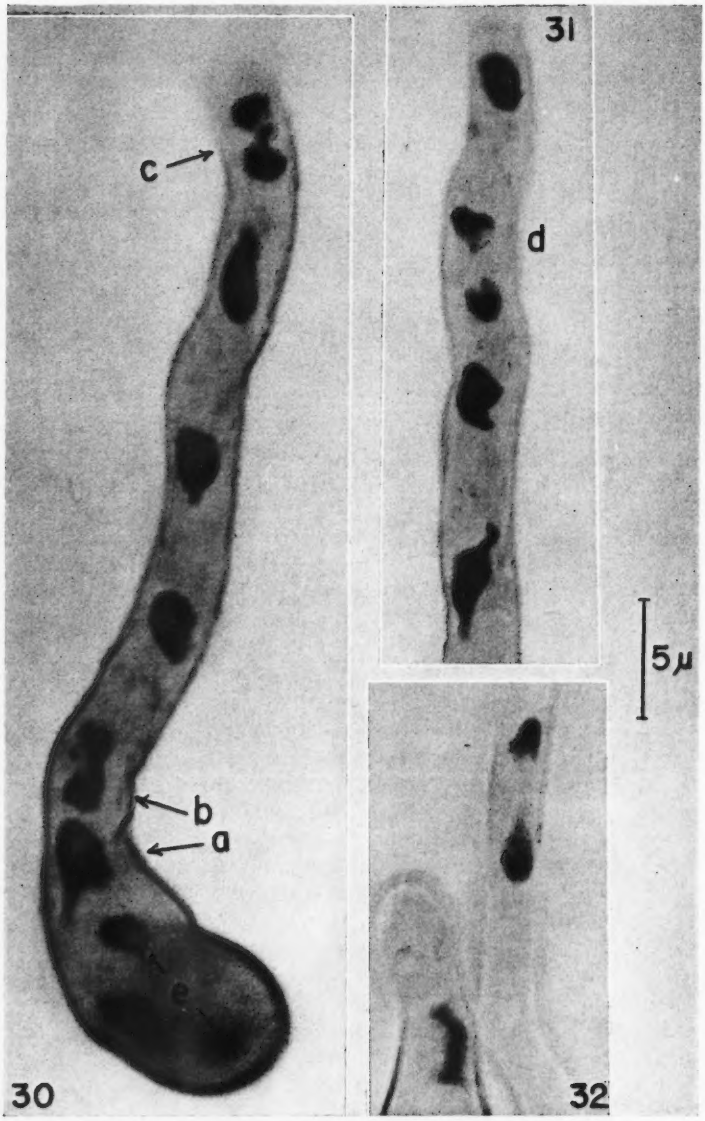


FIGS. 30 and 31. Nuclei at rest and in various phases of direct division in germ tubes grown on cellophane, fixed with acetic acid alcohol, hydrolyzed with *N*/HCl at 60° C., stained with Giemsa solution, and mounted in Gurr's "Giemsa buffer"; *a–e* represent successive stages of nuclear division.

FIG. 32. As above but stained after Feulgen and mounted in acetocarmine. At left bottom an early stage of nuclear division, similar to that shown in FIG. 19, Plate IV. At the top an almost completely divided nucleus.

2. The Reticulum of the Nuclei

The faintly stained strands which radiate from the nucleolus in Sanfelice-hematoxylin preparations are probably narrow passages of matrix material or intrusions of the cytoplasm between the Feulgen-positive elements. To be sure of this it would be necessary to examine the same nucleus first after it was stained with hematoxylin and then again after Feulgen treatment. This has not yet been done. Ziegler (41) has described a network of fine threads in resting nuclei in the oogonium of *Achlya* (Saprolegniaceae). According to him these nuclei divide by mitosis. But only metaphase and anaphase are illustrated by the author. The absence of drawings of prophase stages leaves the relationship of the nuclear net to the chromosomes on the metaphase plate unsettled.

3. The Low Affinity of Feulgen-positive Elements for Hematoxylin

It is strange that *Mucor* nuclei have a kind of chromatin which is readily detectable only by the Feulgen method but this state of affairs is not without precedent. Instances are known in *Plasmodiophora* (19), and in sporozoa (7, 23); and in the cytology of bacteria the same predicament has given rise to a special, empirically developed technology which, as Cassel and Hutchinson (6) have rightly pointed out, stands in need of more thorough cytochemical analysis than it has received until now. The curious combination of chromophobia with Feulgen-positiveness is particularly puzzling since Colette Vendrely (40) has shown that DNA from calf thymus, in combination with histone as well as freed from it, has a strong affinity for iron alum hematoxylin. The consistently negative reaction of fungal nuclei (unpublished results) to the histone test of Alfert and Geschwind (1) is another indication that the composition of vegetative fungal chromatin is unusual and wants analyzing.

4. The Nucleolus

The chemical nature of the nucleolus has not yet been determined but in *Penicillium* it has been shown to be susceptible to digestion with ribonuclease (30). The meaning of the huge size of the nucleolus in germinating spores and in the nuclei of germ tubes is not understood (Figs. 19 and 20, Plate IV). Measurements of its growth from one division to the next and studies of its composition are needed.

5. The Multiplication of the Chromatin

Observations on living hyphae have shown that the nuclei of *M. hiemalis* divide by a process of elongation followed by constriction first described in the Mucorales by Léger (20). All phases of this process can be recognized in fixed and stained preparations. It now remains to describe the behavior of the chromosomes during the division of the nucleus. This is not easy and requires the making of assumptions. Two obstacles stand in the way of a straightforward statement:

(i) Except for a short interval at the close of division, when they are very dense, the nuclei have a grainy structure. The division of these nuclei can not be described in terms of changing states of the chromatin. There is no distinct

state of rest which changes into one of "prophase" which is followed by "metaphase". Here division can only be described in terms of *changes of shape of the whole nucleus.* In the Feulgen-positive grains of the nucleus the chromosomes must somehow be represented but we do not know how individual grains behave during division. We have therefore no evidence that they themselves *are* chromosomes.

(ii) The second difficulty is the apparent absence of mitotic machinery. The process of mitosis is designed to guide towards each daughter nucleus a replica of every member of the complement of chromosomes in the original nucleus. There is no indication that such a delicate adjustment is being attempted in a dividing *Mucor* nucleus. On the contrary, the shell or wreath of chromatin around the nucleolus simply breaks in two. Daughter nuclei do not receive a share of every one of the visible chromatin particles of the original nucleus. They receive only those particles that are in their half of it.

It would be idle to pretend that such behavior could immediately be described as mitosis, but it could be explained by assuming that the chromosomes have divided and sister chromatids have segregated to opposite poles *before* the constriction of the nucleus. The most probable moment for the execution of this maneuver would be immediately after division when the chromatin forms a compact crescent on one side of the nucleus. On this assumption it is clear why the chromatinic part of the nucleus passes without visible signs of reorganization from states of rest to states of division. The reason is that there is no need for a rearrangement if the chromosomes are already divided and at their proper stations before the nucleus begins to divide. In short: It is tentatively proposed that *Mucor* nuclei divide by a form of endomitosis,[4] obscure in detail, which is followed after an interval by the constriction of the nucleus.

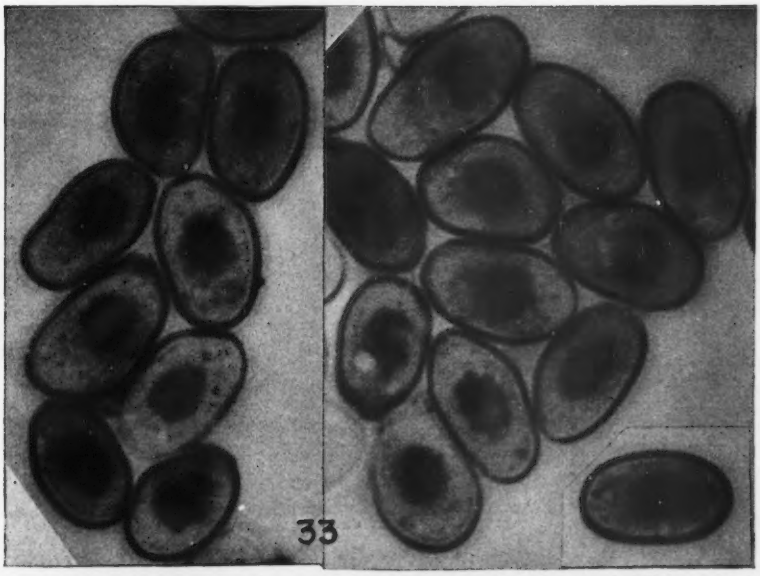
It is a weakness of this assumption that it fails to account for the singleness of the nucleolus in a nucleus regarded as containing two segregated sets of chromosomes. However, the relationship of the chromosomes to the nucleoli in these nuclei remains equally obscure whichever way one looks at it.

Endomitosis may seem a rather farfetched device with which to explain the behavior of *Mucor* nuclei, but it can hardly be said that more conventional concepts have been more successful in this. Whatever the truth about these nuclei may turn out to be, the contemplation of some of the varieties of chromosome behavior known in the protozoa (16) will probably help us to attain it sooner than Procrustes-like loyalty to the canons of classical mitosis. A preliminary note by Girbardt (15), published after this work had been completed, indicates that he has come to similar conclusions regarding the mode of division of vegetative nuclei in basidiomycetes.

6. The Small Bodies Accompanying the Nucleoli

At present it is difficult to make useful suggestions regarding the small, round bodies which are often found by the side of the nucleolus in hematoxylin

[4]Endomitosis is the name of the process by which chromosomes divide and separate inside a nucleus without the aid of a spindle.



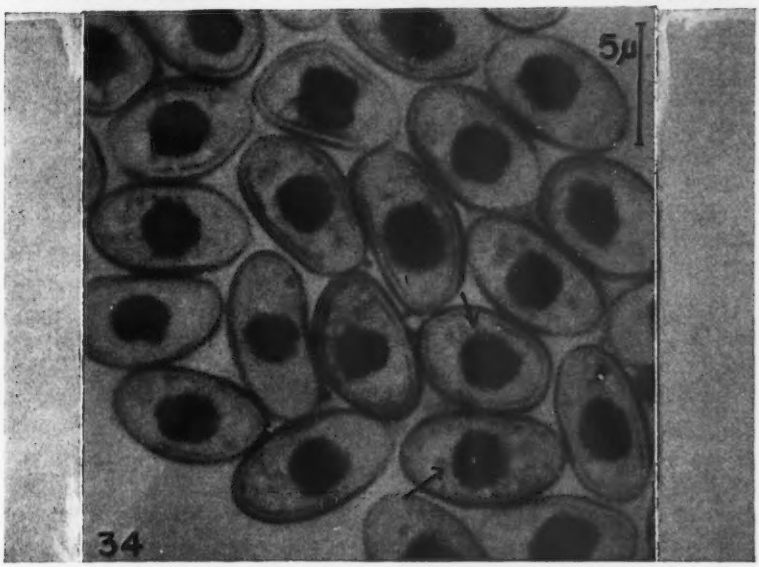
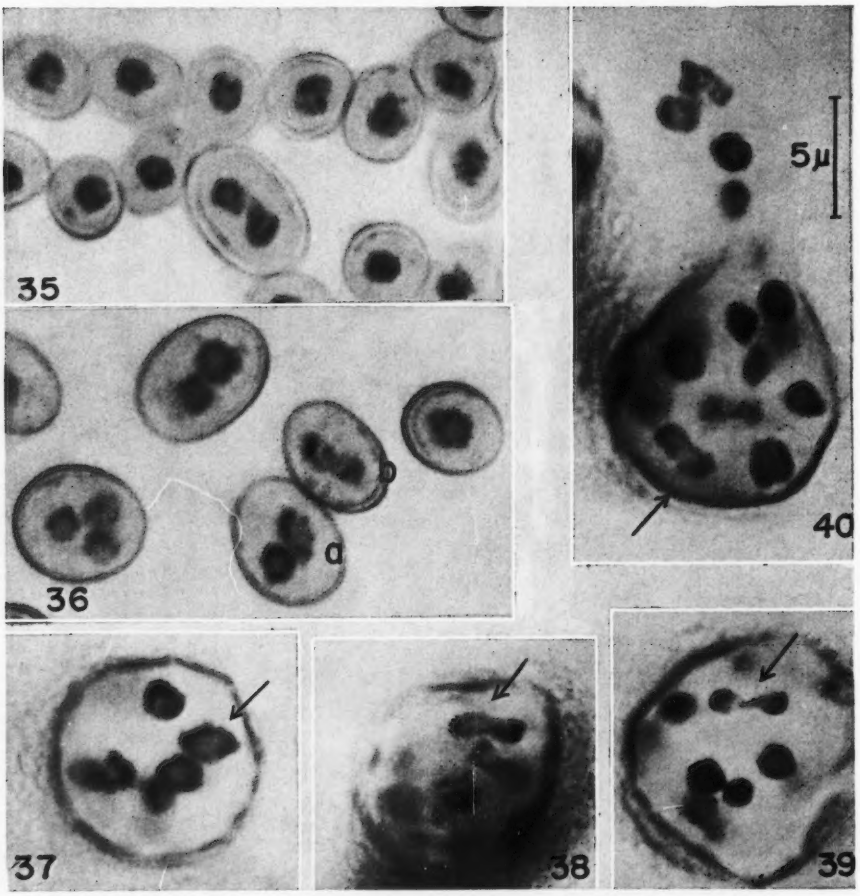


FIG. 33. Resting spores of *Mucor hiemalis*. Fixed with acetic acid alcohol, first stained by the Feulgen procedure, then mounted in acetocarmine. The two halves of this figure as well as the inset at the right bottom corner were part of a single photographic print, cut up and rearranged to make best use of the available space.

FIG. 34. Resting spores from another culture of the same strain of *Mucor*. Acetic acid alcohol, hydrochloric acid, Giemsa, mounted in water. Arrows indicate spores with relatively sharp nuclear detail.

In both preparations the nuclei lack well defined boundaries and are more loosely constructed than the nuclei in growing hyphae.



Mucor fragilis. Note that the magnification is the same as that of the photographs on Plates II–VII.

FIG. 35. Resting spores with spongy nuclei. Acetic acid alcohol, hydrochloric acid, Giemsa.

FIG. 36. Spores fixed and stained as above 1½ hours after transfer to fresh medium. Nuclear division at *a* and *b*. The dividing nucleus at *a* resembles nuclei in the same condition in FIGS. 19 and 21.

FIGS. 37–40. Germinating spores, impression film on fresh egg white, fixed with Newcomer's fluid, hydrochloric acid, Giemsa. Preparation by Mr. R. Pontefract. Arrows point to dividing nuclei.

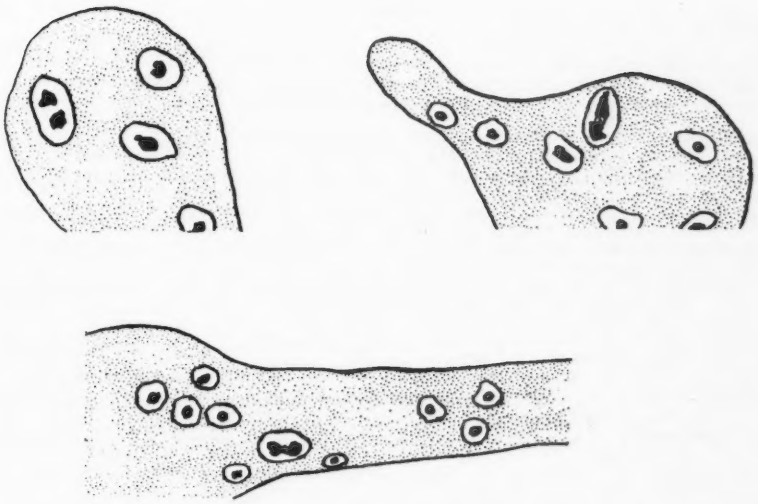


FIG. 41. Nuclei in germinating spores of *Mucor hiemalis*. Carnoy, iron alum hematoxylin (based on Sjoewall, 1945). Explanation in the text.

preparations. It is unlikely that they are centrioles since spindles are not formed during the division of the nuclei. If the bodies represented satellite nucleoli originating in a specific chromosome then the idea of two, genetically identical, sets of chromosomes in the resting nuclei, outlined above, would require that two bodies are found in every nucleus. However, they usually occur singly. We obviously have first to know something about the behavior of these entities during division before we can attempt to theorize about them.

One can now understand why it has so often been held that divisions of vegetative nuclei of Mucorales are hard to find. A drawing of nuclei as they appear in hematoxylin preparations, representative of countless others in the literature, is reproduced in Fig. 41, from the work of Sjoewall (35). There can be little doubt that suitable techniques would have shown that the elongated nuclei with two nucleoli in each of the three groups in this figure were nuclei in division. What has been looked for was a nucleus which divides by mitosis and whose chromosomes can be stained with hematoxylin or gentian violet. The nuclei that were found are not of this kind. They divide directly without spindles or metaphase plates and their chromatin has no affinity for hematoxylin, even at the height of division. The nucleolus persists and is divided between daughter nuclei. This combination of unsuspected properties has concealed the mode of division of *Mucor* nuclei from investigators who have only used traditional methods. There are strong indications that vegetative nuclei of other kinds of fungi behave in a similar manner.

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THE STRUCTURE AND BEHAVIOR OF THE NUCLEI IN SPORES AND GROWING HYPHAE OF MUCORALES

II. PHYCOMYCES BLAKESLEEANUS[1]

C. F. ROBINOW

Abstract

Nuclei of the sporangiospores of seven strains and the growing hyphae of five strains of *Phycomyces blakesleeanus* have been examined in fixed and stained preparations. One strain was found to have unusually large nuclei and has been studied in detail. The nuclei in the sporangiospores consist of Feulgen-positive granules and short filaments in a diffuse matrix. In the ordinary strains the nuclei occur in a narrow range of sizes with their largest diameters around 2 microns. In the spores of the unusual culture the nuclei are of several sizes and many have diameters around 4 and 5 microns.

Similar differences are found between the nuclei in growing mycelia. The nuclei in hyphae resemble those of *Mucor hiemalis*. They consist of a shell of tightly packed granules and filaments of chromatin curving around a relatively large nucleolus. The nuclei divide by elongation followed by constriction. The nucleolus divides at the same time and in the same way and half of it passes to each sister nucleus. The possibility is discussed that the strain with the large nuclei may have had its origin in a spore formed by a germinating zygote and containing both haploid and diploid nuclei. The nuclei of the unusual culture, first examined 3 years ago, have preserved their large size through many cycles of vegetative propagation.

Introduction

In an earlier paper in this Journal (13) the writer has described the nuclei in growing hyphae of *Mucor hiemalis* and *M. fragilis* as consisting of a nucleolus surrounded by a variously shaped shell of closely packed chromatin particles. It was also stated there that the nuclei divide directly by a process of elongation and constriction and that the nucleolus is divided at the same time and in the same way, half of it passing to each sister nucleus. The nuclei in the spores were said to differ from those in growing hyphae by having a loosely knit, net- or sponge-like structure and less sharply defined nucleoli.

The present paper attempts to strengthen the validity of these observations by supporting them with photographs of the much larger nuclei of a special strain of a fellow *Mucor*: *Phycomyces blakesleeanus*.

According to Burgeff (4) *P. blakesleeanus* includes most of the cultures that have been described as *P. nitens* before 1925. Few papers deal with the cytology of these organisms (1, 5, 10, 11, 14, 15) and these, except for a few tantalizing, unillustrated remarks by Burgeff (3) on directly dividing clusters of chromatin granules, contain little useful information on the structure and division of the vegetative nuclei. There is general agreement that the nuclei of *Phycomyces* are very small and resemble those in other Mucorales.

[1]Manuscript received March 5, 1957.
Contribution from the Department of Bacteriology, University of Western Ontario, Faculty of Medicine, London, Canada. This research was supported by a grant from the National Research Council of Canada.

Materials and Methods

1. Organisms

A "plus" and a "minus" culture of each of two strains, Burgeff and Zycha, were obtained from the Centraalbureau voor Schimmelcultures at Baarn, Holland. Another pair, No. 8743 plus and minus, was obtained from the American Type Culture Collection (ATCC), Washington, D.C. The spores of all these have nuclei with diameters of the order of 2 microns. But when spores of descendants of the ATCC strains in the collections of the National Research Council, Ottawa, and the Department of Dairying at the University of British Columbia, Vancouver, still bearing the same number 8743, were examined they were found to contain nuclei of different sizes *including many with diameters of 4–5 microns.* Most of the observations to be described were made on the N.R.C. strain.

2. Methods of Cultivation

On the recommendation of Prof. Johanna Westerdijk of the Baarn Laboratories stock cultures were maintained on rice. The medium most frequently used was composed of:

rice	40 g.
tap water	80 ml.
yeast extract	1.2 g.
Difco "Casamino acids"	1.2 g.

The medium was autoclaved in 500 ml. conical Pyrex flasks, inoculated with spores or tips of growing hyphae, plugged with cotton wool, and covered with aluminum foil. After 2 to 3 weeks sporophores had usually reached the neck of the flask and the culture was then transferred to a refrigerator kept at 4°–6° C. For cytological work and as a check on the purity of the cultures transfers were made to Petri dishes of a medium composed of yeast extract 0.5%, glucose 2%, and agar 1.5%. Sometimes this medium was mixed with equal amounts of the salts – trace metals medium of Hamner, Stewart, and Matrone (8), which was prepared for the author by Dr. C. L. Hannay. I am indebted to Miss Mary T. Clement, Curator of the culture collection of the National Research Council, for useful information on media and the history of No. 8743 N.R.C.

3. Microscopy

Living cultures of *Phycomyces* have been examined with phase contrast microscopy but not as systematically as cultures of *Mucor.* The present paper is chiefly concerned with appearances seen in fixed and stained preparations.

FIG. 1. Spores of *P. blakesleeanus*, strain Zycha-minus, from a culture on rice plus casamino acids plus yeast extract. Newcomer's fixative, Azure A – sulphur dioxide. Wet mount. The magnification, 3600 times, is the same as on the other plates, with the exception of Plate III where it is only 2000 times. The spores contain many nuclei, not all in the same plane of focus. Arrows point to nuclei with fairly well resolved detail. Similar nuclei are in the second and third spore above the number 1 in the left bottom corner. In one of the spores hydrolysis has failed to remove all the basophilia from the cytoplasm.

PLATE I

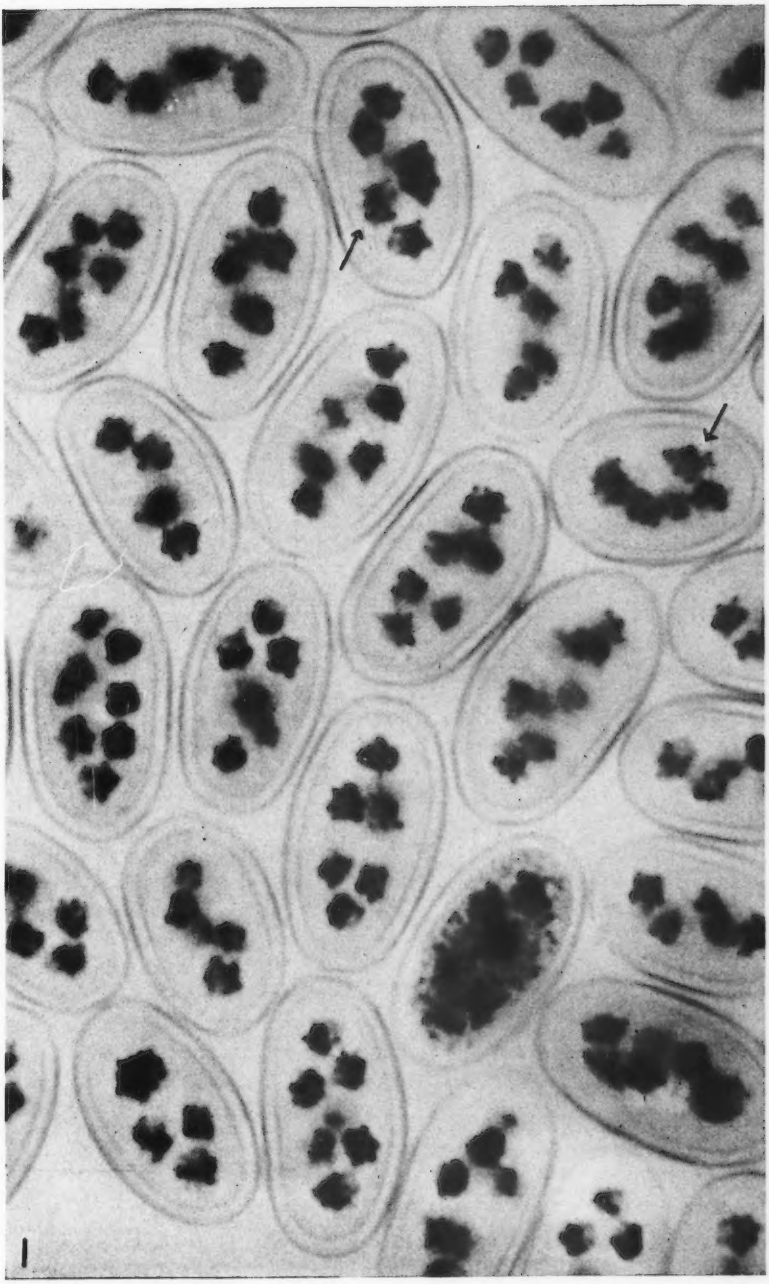

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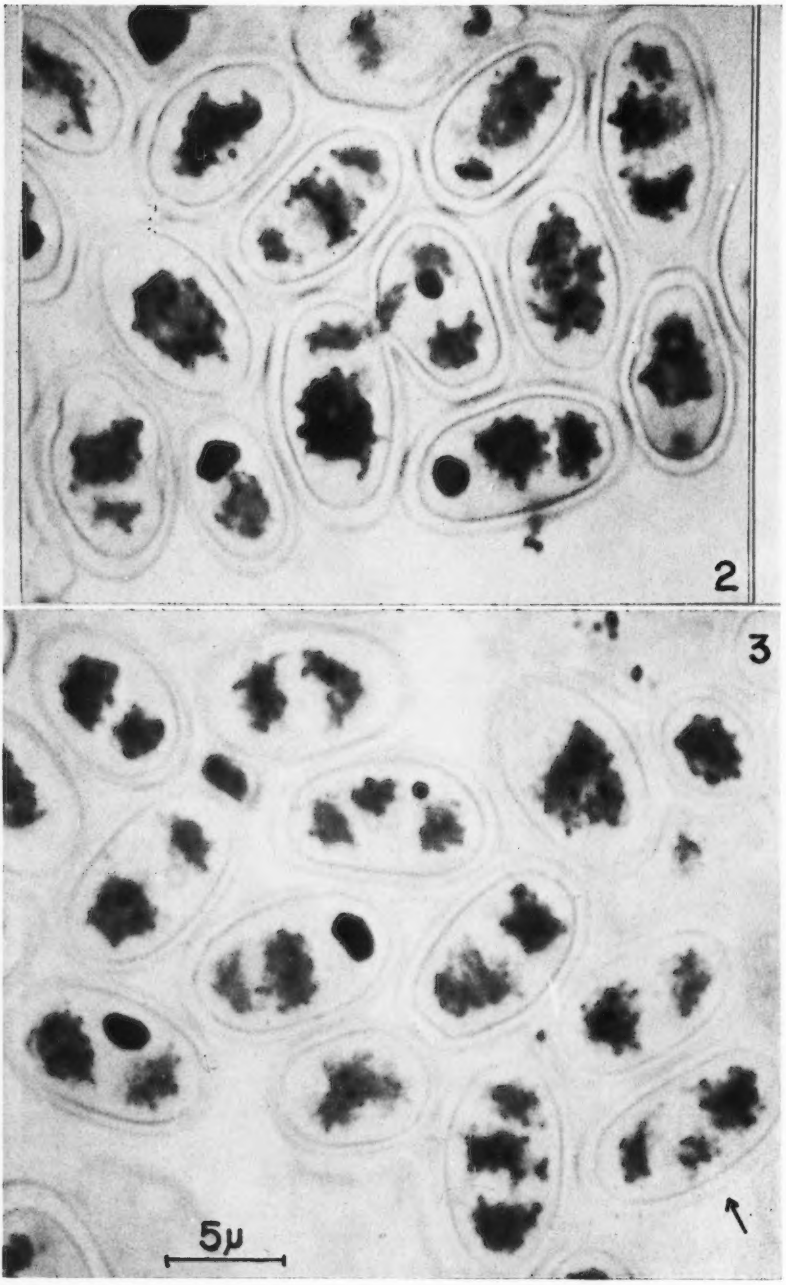
PLATE II



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The most useful specimens were obtained after acetic acid alcohol fixation followed by either the Feulgen or the hydrochloric acid – Giemsa procedures. These two methods have also been used after fixation with osmium tetroxide. Full details of all techniques used are given in the earlier paper on *Mucor*. Comparison with living nuclei of *Mucor* has shown that these techniques are fairly reliable. It is assumed that they can be trusted to give equally useful information on the nuclei of *Phycomyces*.

Spores

An additional procedure, Huebschman's Azure A – sulphur dioxide (9), not mentioned in the earlier study, has yielded very transparent preparations of spores with well stained nuclei. Films of spores prepared and fixed as described for *Mucor* were placed for 6–9 minutes in *N*/1 HCl at 60° C., washed with water, and immersed for 1–2 hours in 10 ml. of 2% Azure A in water in a "Columbia" staining dish[2] to which had been added 0.6 ml. of *N*/HCl and 0.6 ml. of a 10% solution of potassium metabisulphite. Stained films were washed with and mounted in tap water. If necessary they were first differentiated in 95% alcohol.

Hyphae

The Feulgen reaction and the hydrochloric acid – Giemsa method were preferred for the staining of the nuclei in growing hyphae.

Results

A. The Nuclei of Ordinary Strains of Phycomyces blakesleeanus

The nuclei in the stately spores of *Phycomyces* have angular shapes and rough contours. They seem to consist of coarse grains and short filaments of chromatin held together by a diffuse matrix. The size of the nuclei is constant within narrow limits but the average number per spore varies with the composition of the medium in the manner previously described by Huebschman (9) of macroconidia of *Neurospora*.

The spores in Fig. 1 are representative of the spores of all the ordinary strains that I have examined and, incidentally, also resemble those of two strains of true *Phycomyces nitens*, which were received through the kindness of Dr. C. W. Hesseltine.

The spores of the Burgeff and Zycha strains (the ATCC cultures were not germinated) grow into hyphae crowded with innumerable small nuclei of the

[2]Supplied by A. H. Thomas and Co., Philadelphia, Pa.

FIGS. 2 and 3. Spores of *P. blakesleeanus* No. 8743 of the N.R.C. Collection, Ottawa. Acetic acid alcohol, hydrochloric acid, Azure A – sulphur dioxide. Wet mount. The magnification is the same as on Plate I. The nuclei consist of short filaments and granules in a diffuse matrix. Detail is clearest in the spores in the lower right quadrant of FIG. 3. The arrow points to a nucleus which is of the same order of size as many of the nuclei in FIG. 1, Plate I. Several spores contain opaque masses of chromatin which probably represent dying nuclei. There is no suggestion of a nuclear membrane. Two spores in FIG. 2 are connected by a narrow neck. Such connections between neighboring protoplasts are normally severed when the spore walls are laid down. The occasional occurrence of double spores is mentioned by Burgeff (3).

kind illustrated in Fig. 21. The nuclei in the hyphae differ from those in spores by their smoother contours and by having a well marked nucleolus. They resemble the nuclei of *Mucor hiemalis* in every way and, as far as can be judged from fixed preparations, divide directly.

B. The Nuclei of Strain 8743 of the N.R.C. Collection, Ottawa

1. The Spores (Plates II and III)

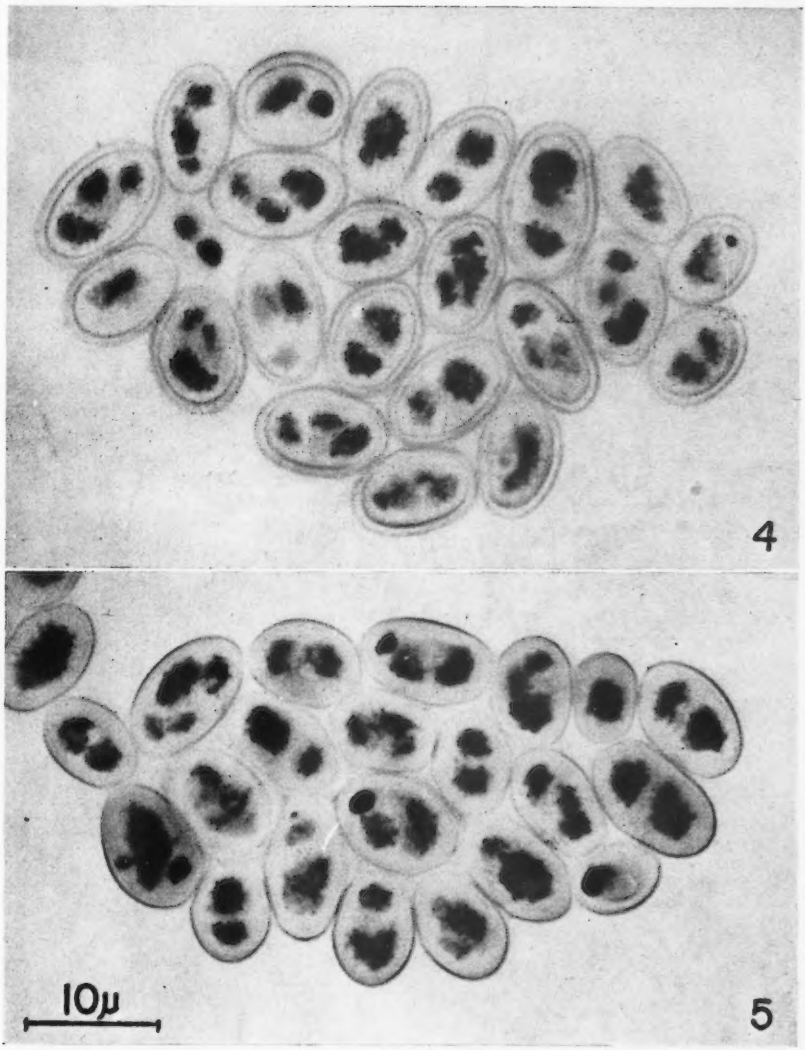
The nuclei in the spores vary greatly in size. The smallest do not exceed the nuclei in the spores of the Zycha strain (Fig. 1) but most of them are much larger. The nuclei can be described in the same terms as the nuclei in spores of *M. hiemalis*, *M. fragilis*, and ordinary strains of *Phycomyces*. They consist of short filaments and granules of chromatin in a diffuse matrix. Filaments frequently protrude above the surface of the nuclei and thereby strengthen the general impression that the nuclei are not contained within a continuous membrane. The sponge-like structure of the nuclei is seen to advantage in Feulgen preparations (Figs. 4 and 5). Some spores contain dense homogeneous masses of chromatin which may be tentatively regarded as degenerating nuclei. These recall similar bodies which Olive (12) and Berliner (2) have described in basidiospores of *Gymnosporangium claviceps*. Between and around the nuclei shiny droplets of lipid are seen, for example in the spore in the right top corner of Fig. 2. Phase contrast microscopy of living resting spores of 8743 N.R.C. has revealed in them the same large irregular nuclei with frayed margins which one sees in fixed and stained preparations. The nuclei of this strain have retained their remarkable features through many transfers, including growth from single spores, and look in recent preparations as they looked when they were first received in the laboratory 3 years ago.

2. Germination

A few hours after transfer to a nutrient medium the spores become round (Fig. 20). The chromatin of the nuclei now acquires a finer texture and is more closely packed. The shape of the nuclei becomes angular and a large and sharply defined nucleolus (a concentration, perhaps, of the formerly diffuse matrix) now makes its appearance (Figs. 6 and 7). During the early phases of this transformation into more conventional shapes the nuclei look very odd, rather as if they were being kneaded and pulled about like lumps of dough. The large nucleus in the germinating spore of Fig. 6 still shows traces of this.

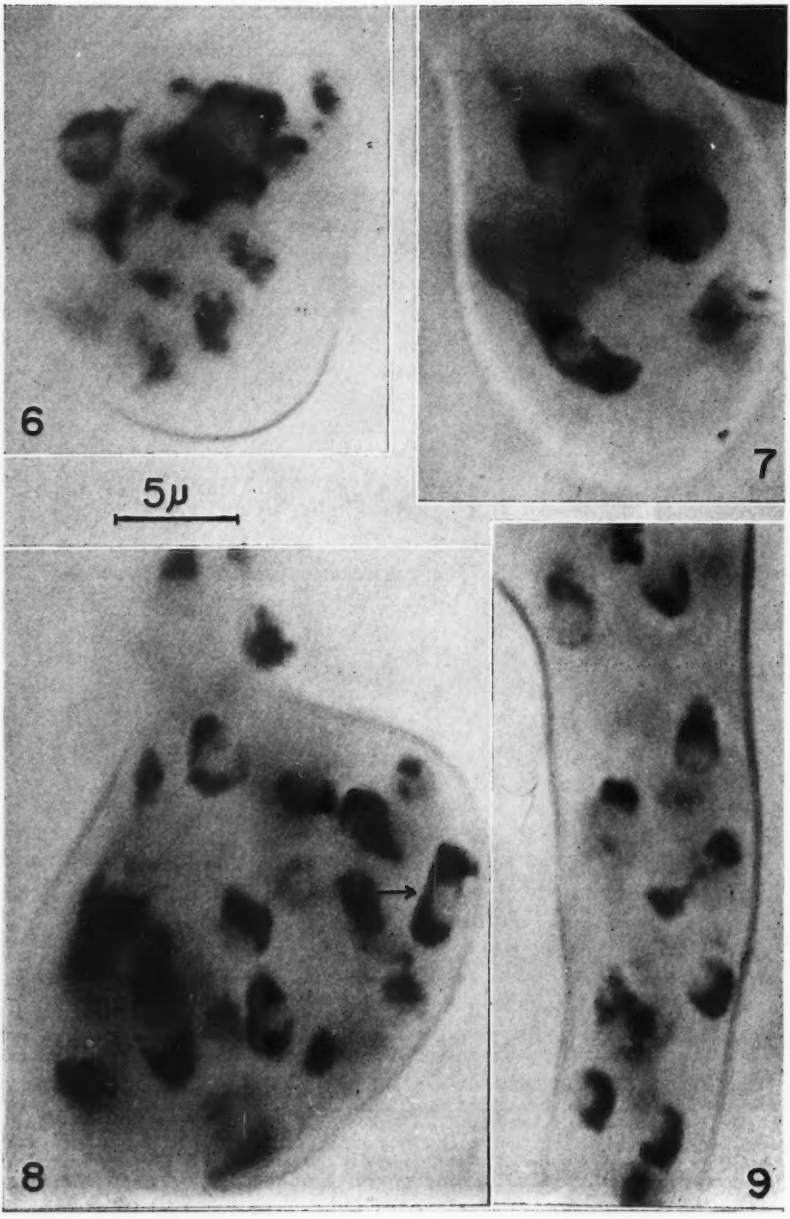
3. The Nuclei in Growing Hyphae (Plates IV, V, and VI)

The nuclei of strain 8743 have essentially the same structure as those of *Mucor hiemalis*. They are amply described in the legends to the photographs and in view of the lengthy description of *Mucor* nuclei in the earlier paper only a few general remarks need be made here. After Feulgen or hydrochloric



FIGS. 4 and 5. Spores of *P. blakesleeanus* No. 8743 N.R.C. Acetic acid alcohol, Feulgen. The spores in FIG. 4 were mounted in water without having been counterstained. Those in FIG. 5 were mounted in acetocarmine. Comparison of the two pictures shows that acetocarmine increases the contrast between nuclei and cytoplasm and reduces the refractility of the spore walls. The nuclei occur in the same range of sizes and have the same open, spongy structures as those in the spores shown on Plate II. The magnification is ×2000.

PLATE IV



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acid – Giemsa staining the main part of the nucleus is crammed with coarse, deeply stained chromatin particles. This high chromaticity of the resting phase is one of the features which nuclei of *Mucor* and *Phycomyces* share with the nuclei of certain protozoa (Dinoflagellida, Euglenida, macronuclei of Euciliatia) (6, 7).

The division of the nuclei has not been studied in living cultures but in fixed and stained preparations many nuclear configurations are encountered which can safely be regarded as states of division because of their close resemblance to the one or the other phase of the process of nuclear division observed in living hyphae of *Mucor hiemalis* (13). On the basis of these experiences it is possible to recognize that the nucleus with straight outer margin and elongated nucleolus, to which the arrow is pointing in Fig. 8, is in an early phase of division. This may also be true of the strongly Feulgen-positive C-shaped nucleus 7 microns to the left of and below the marked one, but this is less certain. Later phases of division are illustrated by Figs. 9, 11, 12, and 19. Configurations resembling mitosis have not been seen and it is concluded that the nuclei of *Phycomyces*, like those of *Mucor* (13) and *Saprolegnia* (16), divide by a process of elongation followed by constriction. The division of the chromatin is usually very accurate (Figs. 9, 12, 17, and 19); whether it is always so may be doubted in view of the wide range of nuclear sizes encountered, particularly in germinating spores. Comparison of the dividing nuclei in Figs. 9, 11*a*, and 11*b* with nondividing nuclei in the same photographs shows that division is not accompanied by obvious changes in the appearance of the chromatin. This makes it difficult to describe division in terms of chromosome behavior. It is only in the last phase of division, illustrated by Figs. 17 and 19, that the chromatin tends to be more than usually dense and homogeneous. In the earlier paper on the nuclei of Mucorales it was tentatively suggested that it is at this stage that each sister nucleus accomplishes the division and initiates the segregation of its chromosomes.

FIGS. 6–9. Germinating spores and a segment of a germ tube of *P. blakesleeanus* No. 8743 N.R.C., 12 hours after having been seeded on cellophane over yeast extract – glucose agar. Acetic acid – alcohol (1: 4), Feulgen, acetocarmine. The magnification is the same for all figures and is indicated by the scale beneath FIG. 6.

FIG. 6. The chromatin of the large lobed nucleus near the top of the figure is in two flat curved sheets like cupped hands which hold the (unstained) nucleolus between them. Outside the plane of the paper a narrow, jagged gap can be dimly seen where the two sheets of chromatin fail to meet on the other side of the nucleolus.

FIG. 7. Vegetative nuclei with tightly packed chromatin sharply set off from a large, round, Feulgen-negative nucleolus. There is a marked difference between these compact angular shapes and the large loosely organized nuclei in resting spores (Plates II and III) which seem to lack a distinct nucleolus.

FIGS. 8 and 9. The arrow in FIG. 8 points to a nucleus about to enter the phase of constriction. Nuclei in this condition bear no resemblance to metaphase plates of mitosis but later stages of division, where the sister nuclei have separated (FIG. 9 center), often look like typical anaphase figures. It should be remembered though that it is not a spindle but the elongated and about-to-divide nucleolus which keeps the nuclei apart in these configurations. Note that the dividing nuclei in both figures differ only in shape but not in texture from neighboring resting nuclei.

Discussion

Burgeff (3) describes the nuclei of *P. blakesleeanus* (unfortunately without showing them) as minute clusters of chromatin particles lacking both a nucleolus and a nuclear membrane. The clusters are said to divide by falling apart into two smaller clusters. One would like to believe that Burgeff was writing of the Feulgen-positive particles of the nuclei but it is unlikely that the hemalum[3] which he used would have revealed them. More probably it was the nucleolus with its strong affinity for hematoxylin which gave Burgeff the impression that he was looking at chromatin. Both chromatin and nucleolus, as the present and the preceding study have shown, do indeed divide "by falling apart". There is therefore a core of truth in Burgeff's findings. Their correct evaluation will have to await a repetition of his experiments. The large size of its nuclei makes strain 8743 of the N.R.C. collection a useful specimen with which to demonstrate the salient features of mucorine nuclei and it is from this point of view that the culture has been discussed in the present paper. But the strain presents also special problems of its own. Why does it have nuclei of different sizes and why are many of them very large? There are probably other strains of *P. blakesleeanus* with both large and small nuclei and it is to be hoped that this communication will stimulate a search for them. A possible origin for such cultures is suggested by cytological observations of Burgeff (3, p. 359), Cutter (5), and Sjoewall (15) on the behavior of the nuclei in germinating "germ sporangia" (the sporangia that arise from germinating zygospores) of *P. blakesleeanus*. Burgeff states that the sporophore contains very large nuclei with nuclei measuring 6–7 μ in diameter. The large nuclei enter mitosis when the sporangium is beginning to be formed. "The exact course of mitosis is difficult to follow", writes Burgeff, "only prophase, which involves loss of the nucleolus, was readily identified". Cutter (5) finds nuclei of two sizes in a portion of the germspores and believes that such spores may give rise to mycelia in which: "further sexual segregation occurs at a later date". Sjoewall's findings (15) go some way towards confirming Cutter's observations and are in good agreement

[3]Cutter (5) criticizes Burgeff for having relied only on "iron-alum hematoxylin". This is a mistranslation. Burgeff (3, p. 356) has "Haemalaun", a direct stain used without a mordant and less liable than iron alum hematoxylin to cause misinterpretations.

Nuclei in hyphae of young mycelia of *P. blakesleeanus* No. 8743 N.R.C. All photographs are of wet mounted preparations, fixed in acetic acid alcohol. The magnification of all figures is the same, ×3600, and is indicated by the scale in FIG. 13.

FIG. 10. Hydrochloric acid – Giemsa. Note the varied shapes of the nuclei and their widely differing sizes.

FIGS. 11*a*, *b*. Feulgen, acetocarmine. Two photographs of the same group of nuclei taken at different levels of focus. The nucleus indicated by the arrow is in an advanced state of division. Its nucleolus is elongated and about to be constricted. The chromatin forms a canoe-shaped crest at one side of the nucleolus. Note that the granules of chromatin of the dividing nucleus do not look different from those in the resting nucleus immediately beneath it.

FIG. 12. Hydrochloric acid – Giemsa. Division of chromatin and nucleolus has been accomplished. A narrow bridge of nucleolar material still connects the sister nuclei.

FIG. 13. Feulgen. Irregularly shaped nucleus with large nucleolus. The chromatin has the texture of a coarse net.

PLATE V

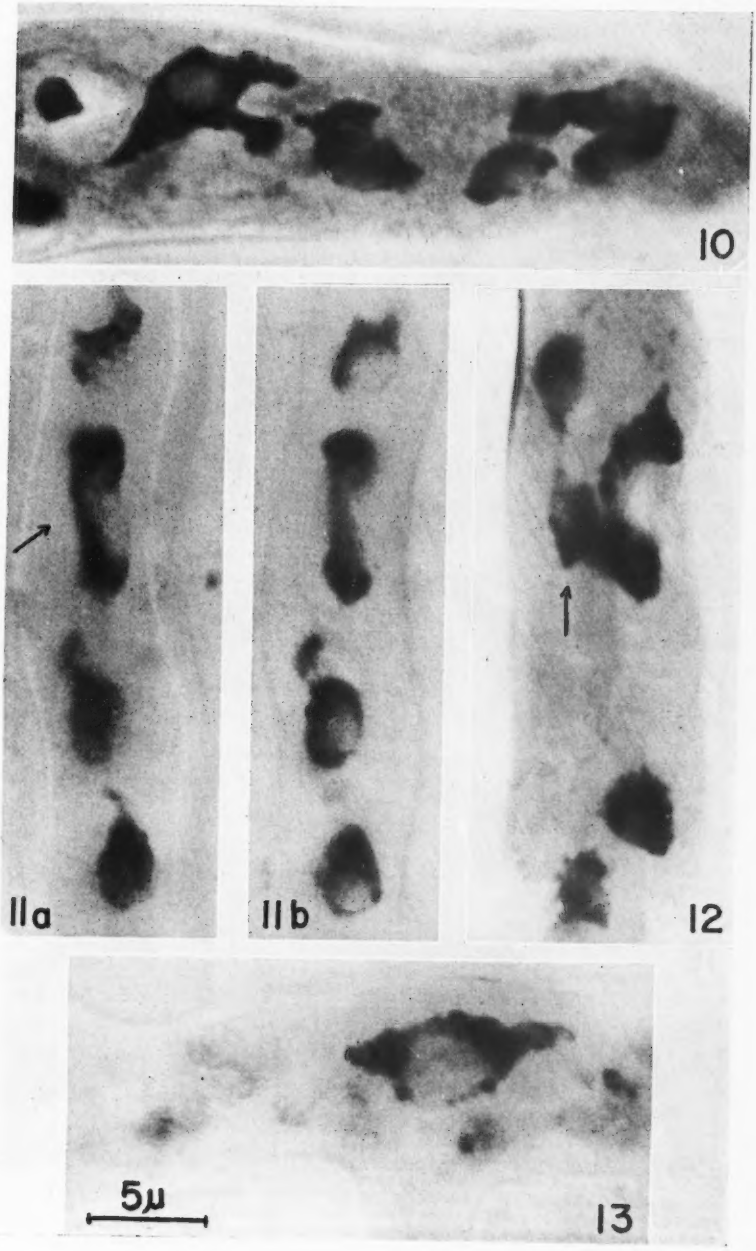
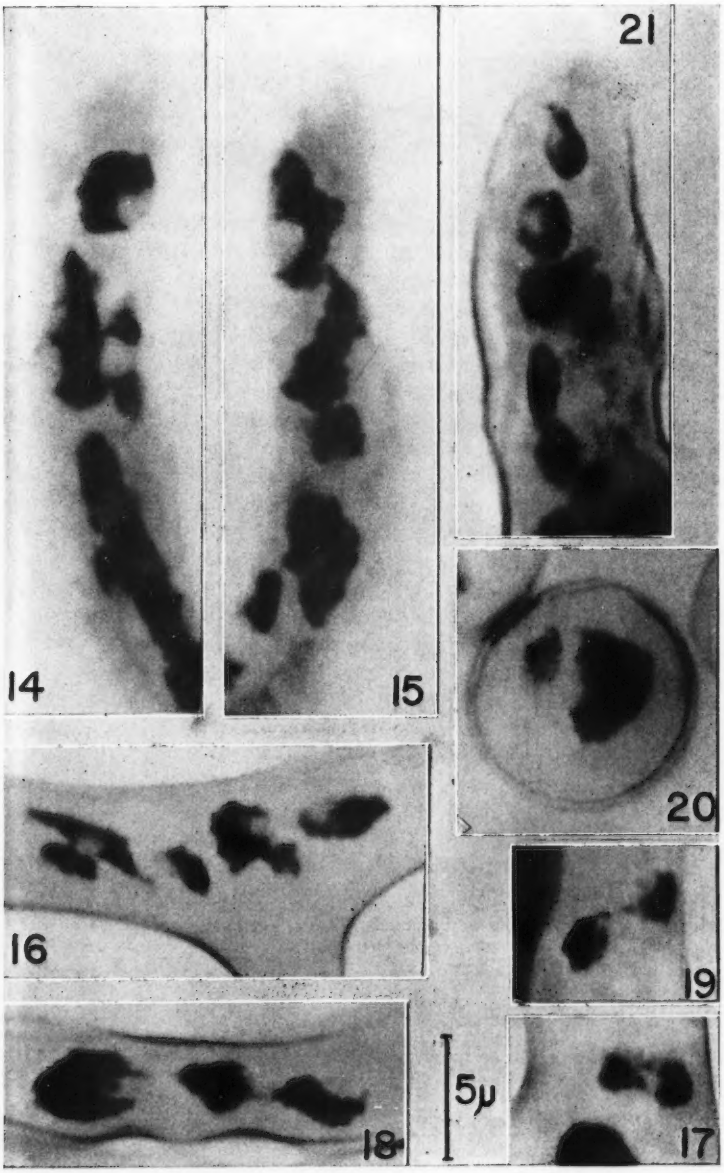


PLATE VI



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with those of Burgeff. In the tip of the growing sporophore Sjoewall found large resting nuclei side by side with very small ones. When the former, which he regards as diploid, enter the developing sporangium they lose their nucleolus and assume a granular condition which Sjoewall suspects of being the prophase of an intended but never consummated reduction division.

Burgeff gives no illustrations and Sjoewall's drawings are schematic but it seems probable that the nuclei in the germ sporangium which the two authors regard as being in prophase were really at rest and in the same state as the nuclei in sporangiospores of No. 8743 N.R.C., with chromatin in the shape of filaments and granules and the nucleolus indistinguishable from a diffuse matrix that extends through the whole nucleus. My findings in *Phycomyces*, and to some extent in *Mucor*, indicate that such is *the normal state of rest* of the nuclei in sporangiospores. It is a special state encountered only in spores and very different from any of the phases of the nuclei in growing hyphae.

Sjoewall (15) saw no further changes of the strange nuclei in germ-sporangia and he thinks it likely that only the spores with small (haploid ?) nuclei are viable and that those that are formed around the large "arrested prophase" nuclei do not germinate. Perhaps a germ-spore containing both large and small nuclei can germinate after all and it was such an event that gave rise to strain 8743 N.R.C. The peculiarity of this culture would then consist in having maintained a heterogeneous assortment of nuclei including, perhaps, diploid ones, through many cycles of vegetative propagation.

The sexual behavior of such a mycelium, as Cutter (5) and others before him have reasoned will be determined "by the balance between the different nuclei present in it". No systematic experiments along these lines have been performed with the problematic culture but its behavior towards one of the

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FIGS. 14–20. *P. blakesleeanus* No. 8743, N.R.C.

FIG. 21. *P. blakesleeanus* strain Zycha, minus.

FIGS. 14 and 15. Eighteen-hour mycelium. Osmium tetroxide, hydrochloric acid, Giemsa. Dehydrated with acetone–xylene mixtures and mounted in DPX artificial resin. Tips of growing hyphae crowded with nuclei of various shapes and sizes. In most of the nuclei the nucleolus is visible as an unstained circular patch, but in the second nucleus from the top in FIG. 15 it is out of focus. Comparison with wet mounts of hyphae fixed in acetic acid alcohol (Plates IV and V) shows that the osmium-fixed nuclei in FIGS. 14 and 15 have shrunk little during dehydration. Note also that the texture of the chromatin is very similar in the two kinds of preparations.

FIGS. 16–19. Eighteen-hour mycelium. Acetic acid alcohol. Hydrochloric acid, Giemsa, DPX. The hyphae and nuclei in this preparation have probably suffered some shrinkage.

FIG. 16. A group of nondividing nuclei of various shapes and sizes.

FIGS. 17, 18, and 19. These illustrate three phases of nuclear division. Compare with FIGS. 11*a*, 11*b*, and 12, Plate V.

FIG. 20. Acetic acid alcohol, hydrochloric acid – Giemsa. Wet mount. Early stage of germination. Compare with resting spores in FIGS. 2 and 3, Plate II, and note that germination is accompanied by closer packing of the chromatin and the emergence of a clear cut nucleolus.

FIG. 21. *P. blakesleeanus*, strain Zycha, minus. Osmium tetroxide, hydrochloric acid, Giemsa. Dehydrated and mounted in DPX. Comparison of the nuclei in this figure with those of strain 8743 in the comparable treated preparation of FIGS. 14 and 15 suggests, and the study of larger samples confirms, that there is some overlap of size classes between the two strains but that the normal nuclei occur in a much narrower range of sizes and are, on the whole, considerably smaller than those in strain 8743. The hypha shown in this picture was grown from spores of the kind illustrated in FIG. 1, Plate I.

normal Zycha strains is in keeping with the expectation of impaired sexuality. Three mycelia, Zycha plus, Zycha minus, and 8743 N.R.C., were confronted on the same agar medium. Numerous black zygospores developed along the line where the plus and minus mycelium were in contact but only very few pale and imperfectly ornamented ones were formed along the line of contact between Zycha-minus and 8743 N.R.C. A comparison with its own normal namesake, 8743 ATCC, has not yet been made.

A fresh study of the nuclear events during the formation and the germination of the coeno-zygotes of *Phycomyces* is now to be wished for.

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An abstract of not more than about 200 words, indicating the scope of the work and the principal findings, is required, except in Notes.

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